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Characterisation of *oskar* 3'UTR RNA domains involved in early RNA localisation and translational repression.

Examiners: Prof. Dr. Matthias Hentze und Prof. Dr. Eduard Hurt

Contents

Abstract

Induction of *D.melanogaster* germ cell formation depends on local activity of the maternal determinant Oskar at the posterior of the embryo. When Oskar is mis-expressed at the anterior, posterior structures develop ectopically, while absence of Oskar leads to loss of abdominal structures and germ cells. Spatial restriction of Oskar protein is achieved by enriching *oskar* mRNA in the oocyte, localising it to the posterior pole and by maintaining the mRNA translationally silencing during transport. The 3'UTR of *oskar* RNA contains elements for its translational repression and is, together with splicing, also required for posterior localisation. Intronless reporters bearing the *oskar* 3'UTR can also localise, however this is an indirect process involving hitch-hiking of the reporter RNA with endogenous *oskar* mRNA, to the posterior pole.

We have analysed the molecular basis of the hitch-hiking process and tested its potential implication in regulation of endogenous *oskar* RNA *in vivo*. *In vitro* the *oskar* 3'UTR RNA forms RNA-RNA dimers via a specific RNA dimerisation domain. *In vivo*, this dimerisation domain is necessary - though not sufficient - for efficient hitch-hiking of 3'UTR-containing reporters with endogenous *oskar* mRNA to the posterior of the oocyte. In contrast, the dimerisation domain is not essential for *oskar* mRNA localisation. Surprisingly however, offspring of females expressing *oskar* RNA mutated in its dimerisation domain display patterning defects suggesting Oskar protein over-expression. Consistent with this, we found that in this *oskar* mutant the mRNA is prematurely and ectopically translated. This ectopic translation is suppressed when dimerisation is restored by co-expressing an *oskar* RNA bearing compensatory mutations that *in vitro* restore RNA dimerisation. My work thus revealed a direct role for RNA-RNA interaction in translational repression of *oskar* mRNA.

Zusammenfassung

Während der Oogenese und frühen Embryogenese von *D. melanogaster* bestimmt die lokale Aktivität des maternalen Oskar-Proteins, die Induktion der posterioren Strukturen Abdomen und Keimbahn. Fehlt Oskar-Aktivität, kann dies zu einem kompletten Verlust der posterioren Stukturen führen. Umgekehrt führt die Aktivität von Oskar-Protein am anterioren Pol zur Entwicklung von ektopischen Keimbahn und Abdomen. Verschiedene Mechanismen stellen eine räumlich begrenzte Aktivität des Oskar-Proteins sicher: Zunächst wird *oskar*-mRNA in der Eizelle angereichert und innerhalb der Eizelle anschließend am posterioren Ende konzentriert. Außerdem wird die Translation von noch unlokalisierter mRNA unterdrückt. Für diese Regulationsmechnismen ist der 3'UTR der *oskar*-mRNA essentiell. Für die Lokalisierung der mRNA am posterioren Pol ist außerdem das Spleißen an der Intron-Position 1 nötig. Allerdings wurde beobachtet, dass über eine indirekte Art der Lokalisierung Reporter-RNAs, die nur den *oskar*-3'UTR enthalten, ebenfalls den posterioren Pol erreichen können.

In der vorliegenden Arbeit habe ich die molekulare Basis dieses indirekten Transportprozesses und seine mögliche Implikation für die Regulation von endogener *oskar*-mRNA *in vivo* untersucht. *In vitro* kann die *oskar*-3'UTR-Sequenz über eine RNA-Sekundärstruktur intermolekulare Dimere bilden. Ich konnte zeigen, dass die Dimerisierungsdomäne *in vivo* tatsächlich an den indirekten Transportprozessen der 3'UTR-Reporter-RNAs innerhalb der Eizelle beteiligt ist. Für den aktiven Transport von endogener *oskar*-mRNA ist die Dimerisierungsdomäne hingegen nicht nötig. Überraschenderweise zeigten jedoch Embryos, welche die mutierte Version der *oskar*-mRNA tragen, Musterbildungsdefekte, die auf eine Überexpression des Oskar-Proteins hindeuten. Tatsächlich konnte ich sowohl in den Embryos als auch schon in den Oozyten der Mutanten erhöhte Menge an Oskar-Protein nachweisen und außerdem zeigen, dass das Oskar-Protein in der Entwicklung ektopisch und verfrüht translatiert wird. Als nächstes habe ich zwei mRNAs zeitgleich exprimiert, die zueinander komplementäre Mutationen in der Dimerisierungsdomäne aufweisen. *In vitro* erlauben diese Mutationen eine Wiederherstellung der Dimerisierung. Auf diesem Weg konnte ich die embryonalen Fehlbildungen fast vollständig unterdrücken. Zusammenfassend konnte ich zeigen, dass die Interaktion von *oskar*-mRNAs *in vivo* direkt an der Unterdrückung der Translation beteiligt ist.

1. Introduction

1.1. *D.melanogaster* as a model organism

1.1.1. Historical perspective

The fruit fly *Drosophila .melanogaster* was introduced to experimental biology one hundred years ago and the first discovery of a heritable mutation was published in 1910 by T.H. Morgan (Morgan 1910).While for many years genetics of *D.melanogaster* was the main focus of research, recently the fly proved to also be an invaluable tool to study genomics, neurobiology and is today also used as a model for human diseases (Botas 2007; Stark et al. 2007; Vosshall 2007). What attracts researchers is the easy handling of the fly in the laboratory and a very short generation time.

1.1.2. *D.melanogaster* life style: live fast, die young

Outside the laboratory flies consume readily available supplies like decaying plant or fungus material, that due to their decomposition are available for a limited time. The female fly nurtures and also deposits its eggs into this material, providing the hatching larvae with an immediate source of nutrients. Possibly in adaptation to the fast decay of nutrients, D.melanogaster development is comparatively fast (Figure 1A). A fertilised egg hatches after 24 hours1 and after another four days the larva develops into the pupae stage. After fours days

[1] At optimal conditions at 25°C.

of metamorphosis the adult fly ecloses. As for many ectothermic organisms, also the fly's development from egg to adult is temperature dependent (reviewed in Ashburner, 1989).

1.1.3. Fast embryogenesis accelerates development

The *D.melanogaster* development from egg to larva on average is completed in 24 hours. 15 minutes after fertilisation the egg and sperm pronuclei fuse to form the zygotic nucleus (reviewed in Ashburner, 1989). The egg then undergoes a superficial cleavage that is typical of most insects, due to their large yolk content in the centre of the egg. Nuclear divisions one to eight are synchronised and are completed in just over one hour, therefore each mitosis on average takes eight minutes. The single nuclei then migrate to the periphery and undergo another four divisions, after which the first cell membranes begin to form. For comparison, the first eight mitoses in the zebrafish embryo require three hours for completion, while in early mouse development, 12 to 24 hours pass for each cleavage (Kimmel et al. 1995); Gilbert, 2000). Such rapid nuclear divisions can only be achieved without cell divisions and without transcription of the dividing nuclei, yet already seven hours after fertilisation, gastrulation essentially ceases and the organogenesis, segmentation and imaginal disc formation is completed. To allow this rapid embryogenesis, fly development employs specialised mechanisms described below.

In the first two to three hours after fertilisation, zygotic genes are not transcribed yet RNA evidence of gradients that have crucial functions in organising of the primary body axes was detected in the early embryo. These mRNA based gradients could therefore not be of zygotic, but must be of maternal origin (Kandler-Singer and Kalthoff 1976). The first RNA based gradient described is formed by the homeodomain-transcription factor Bicoid and it was discovered that *bicoid* RNA is localised at the anterior of the early embryo and the protein then diffuses from this source. Since then, more maternally derived mRNAs have been shown to organise early embryonic development in *D.melanogaster*, and thereby allow rapid patterning independent of zygotic transcription. Thus the anlage of the main body axes of the fly in the first hours after fertilisation is not under zygotic but rather maternal control.

1.1.4. *D.melanogaster* oogenesis

Basing early embryogenesis so strongly on maternal RNA contributions requires a specialised type of egg in which many mRNAs are stored. How are these eggs formed? *D.melanogaster* has paired ovaries that develop during metamorphosis and contain multiple strings of

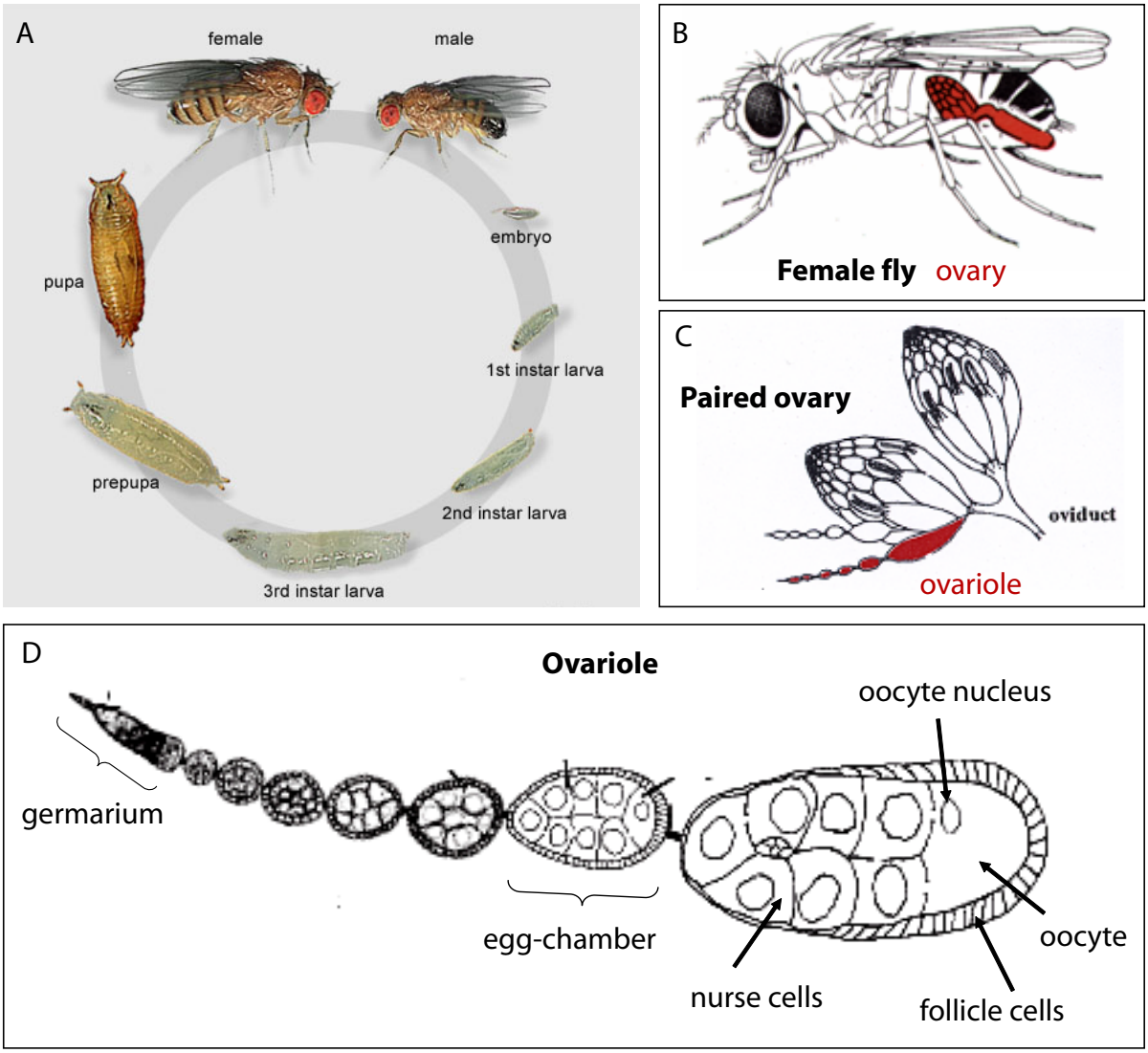


Figure 1: The *D.melanogaster* life cycle and the female reproductive organs.
A. The life cycle of *D.melanogaster*. Embryogenesis begins as soon as the egg is fertilised and ends with the hatching of the larva. After several larval stages, pupation begins and metamorphosis occurs in the pupal case, from which the adult fly emerges (Image from Flymove). B. A schematic of a female fly and its paired ovary shown in red. C. Diagram of the ovaries. Each ovary contains approximately sixteen ovarioles of which one is highlighted in red (B and C courtesy of J.B.Coutelis). D. Detailed scheme of one ovariole. The germarium, left, contains stem cells and a cystoblast. The cystoblast divides into 16 cells that, together with a surrounding layer of epithelial follicle cells, form the egg-chamber. Because of an incomplete cytokinesis, these 16 cells remain interconnected via cytoplasmic bridges. Of the 16 cells, one cell develops into an oocyte, while the others become nurse cells. Due to the continuous production of egg-chambers, each ovariole contains egg-chambers of all developmental stages (modified from Keyes and Spradling, 1997).

ovarioles (Figure 1B,C). An ovariole contains a stem cell niche ("germarium") where oocytes are continuously produced, and oocytes of all developmental stages. In the germarium the stem cell divides asymmetrically to produce another stem cell and an oogonium, which then undergoes four rounds of mitotic divisions. The 16 cells from these divisions remain in a syncytium and are interconnected by cytoplasmic bridges (ring canals). Via these ring canals, cytoplasmic components are transported between neighbouring cells. The syncytial cells are surrounded by an epithelial layer of somatic follicle cells that have crucial functions in oogenesis and axis formation. One germ cell will become the oocyte and the remaining 15 develop into nurse cells (Compare also Figure 1D). Late in oogenesis the nurse cells undergo apoptosis and allow the oocyte to mature into a fertilisable egg.

Autoradiographic analyses revealed that during most of oogenesis active transcription is restricted to the nurse cells, while protein translation also occurs in the oocyte cytoplasm (King and Burnett 1959)Bier, 1963). The contents of the oocyte, including the maternal mRNAs (maternal determinants) that control embryo patterning are deposited from the oocyte into the fertilised egg (Spradling 1993). In consequence the mRNAs of the oocyte, including the maternal determinants, originate from nurse cell transcription (Spradling 1993). Further the asymmetric distribution of maternal determinants in the early embryo is already determined by the subcellular localisation of their RNAs in the developing oocyte. Therefore, the main body axes are preformed in the unfertilised egg and in fact are already morphologically visible in the shape of the egg. In addition to providing the maternal determinants, the nurse cells also supply the oocyte with proteins and RNAs crucial for the progression of oogenesis.

Although meroistic oogenesis is a specialised way of making an egg, it shares molecular components with germ cell formation in other organisms (Tanaka et al. 2000). Additionally, the cellular mechanisms involved in *D.melanogaster* oocytes are re-employed in the organisation and polarisation of other cell types (Roegiers and Jan 2000; Du et al. 2007).

1.2. Local protein activity

1.2.1. The functions and mechanisms of local protein activities

mRNA localisation followed by local translation is used by cells to restrict protein activity. For example the asymmetric cell division of haploid *Saccharomyces cerevisiae* cells depends on localised *ASH1* mRNA. *ASH1* mRNA encodes a transcriptional regulator that represses the expression of genes essential for mating type switching (Bobola et al. 1996). During mitosis,

the *ASH1* transcript is segregated preferentially to the daughter cell, ensuring that Ash1 protein is only expressed in this cell. Consequently, mating type switching is inhibited in the Ash1 expressing daughter cell. Thus, localising *ASH1* mRNA ultimately ensures that mother and daughter cell adopt different mating types.

Cells also use mRNA localisation mechanisms to adopt asymmetric cell shapes. *β-actin* mRNA is localised to the leading lamellae of migrating chicken muscle cells (Lawrence and Singer, 1986, Kislauskis, 1993). This localised mRNA allows the accumulation of high concentrations of Actin in the cell protrusion, a prerequisite for the fast reorganisation of the cytoskeleton during cell movements.

Finally, mRNA localisation is involved in establishing and maintaining intracellular asymmetries and intracellular morphogen gradients. In the Xenopus embryo, *cyclinB* mRNA is localised to and translated at the spindle and the local protein activity is necessary for progression of normal mitosis (Groisman et al. 2000). In *D.melanogaster* the maternal determinants are encoded by localised mRNAs that set up the body axes of the embryo. Several mechanisms have been proposed to explain subcellular mRNA enrichment (Lipshitz and Smibert 2000). Evenly distributed mRNAs can accumulate in one localisation by regional protection from degradation as for example shown for *hsp83* (Ding et al. 1993; Bashirullah et al. 1999). On the other hand mRNAs can become trapped to a spatially restricted receptor such as *nanos* mRNA that initially is dispersed in the oocyte (Forrest and Gavis 2003). However, the majority of localised mRNAs described to date, including the *D.melanogaster* maternal determinants, accumulate by directed transport along the cytoskeleton to a specific intracellular site. The example of *oskar* mRNA will be discussed in more detail below.

1.2.2. Localised mRNAs in *D.melanogaster* oogenesis

D.melanogaster relies on mRNA localisation for its early development and a number of mRNAs show specific subcellular accumulations already in the germ cyst.

In early oogenesis, as shown in Figure 2A, *staufen*, *BicD, tudor, otu*, *cyclinB* and *dacapo* mRNAs enrich in the developing oocyte and are present only at low levels in the nurse cells, where they are initially transcribed (Suter et al. 1989; Golumbeski et al. 1991; St Johnston et al. 1991; Dalby and Glover 1992; Tirronen et al. 1995; de Nooij et al. 2000; Sadusky et al. 2004). Due to the syncytial character of the egg chamber, the oocyte can already be considered as a specific site. The proteins encoded by these mRNAs are required in the oocyte but are not essential, or even harmful if continuously expressed in the nurse cells, as in the

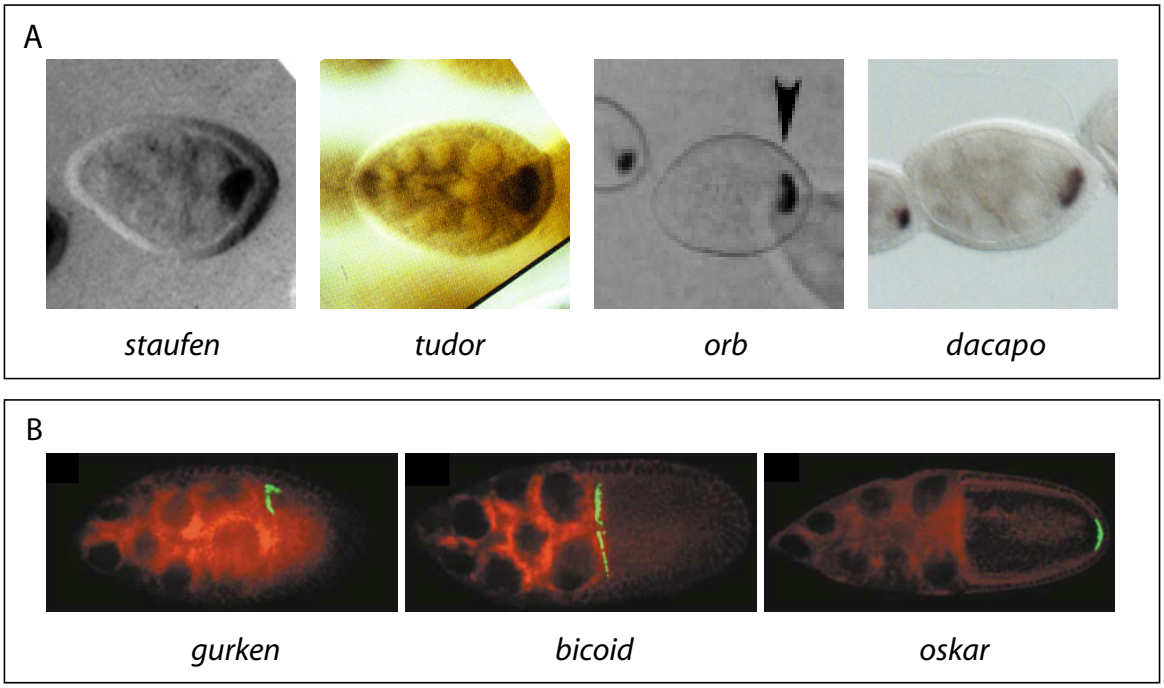


Figure 2: mRNA localisation in *D.melanogaster* **oogenesis.**
A. Several mRNAs have been shown to enrich in the oocyte of stage 6/7 egg-chambers. Shown here are *staufen, tudor, orb and dacapo* mRNAs (StJohnston, 1991; Golumbeski, 1991; Lantz, 1992; deNooij, 2000). B. Localisation of the maternal determinants (green) at stage 10 of oogenesis. While *gurken* mRNA enriches at the anterior-dorsal corner of the oocyte, *bicoid* accumulates at the anterior, and *oskar* mRNA at the posterior pole (modified from Hachet and Ephrussi, 2001). All egg-chambers are oriented with their anterior towards the left and the posterior towards the right.

case of *dacapo*. Other mRNAs localise to specific areas within the oocyte: *fs(k10)* mRNA enriches in the oocyte and is detectable at the anterior pole from mid-oogenesis onwards (Cheung et al. 1992). This early oocyte accumulation of *fs(k10)* mRNA is necessary for the deployment of K10 protein activity (Prost et al. 1988).

The mRNAs encoding maternal determinants also localise within the egg chamber and the spatially restricted activity of the proteins they encode is a prerequisite for proper axis formations (Figure 2B). *gurken* mRNA enriches first in the young oocyte and then predominantly at the anterior-dorsal corner from stage 8 onwards (Neuman-Silberberg and Schüpbach 1993). The Gurken protein, a TGF-α family member, is translated already in early ovarian development when it determines the fate of posterior follicle cells. This is necessary for oogenesis progression and is critical for the definition of the anterior-posterior axis of the future embryo (Gonzalez-Reyes et al. 1995; Roth et al. 1995). Shortly thereafter Gurken is translated at the anterior-dorsal corner of the oocyte, where it signals to the most proximal follicle cells overlaying the cyst. This second signalling event induces and defines the position of the dorsal appendages micropyle and aeropyle (Schupbach 1987; Neuman-Silberberg and Schüpbach 1993). In summary Gurken protein is involved in determining both the anterior-posterior and the dorso-ventral axes of the embryo.

Similar to the *gurken* message, *bicoid* mRNA accumulates in the early oocyte (stage 5-6), then enriches at the anterior cortex from stage 8 of oogenesis onwards (Berleth et al. 1988; St Johnston et al. 1989). In contrast to Gurken, Bicoid protein is translated only after fertilisation, when it induces the formation of head structures and simultaneously suppresses posterior structures at the anterior pole of the embryo (Frohnhofer et al. 1986; Nusslein-Volhard et al. 1987; Driever and Nusslein-Volhard 1988; Driever et al. 1990; Salles et al. 1994).

oskar mRNA, discussed in detail in the following section, also localises to the young oocyte. From stage 9 onwards, the RNA accumulates at the posterior pole and this site of RNA enrichment is specific to *oskar* mRNA at this stage. Oskar protein is translated already during oogenesis, and its activity is required to define posterior structures and for inducing germ cell formation.

Thus, with so many localised mRNAs described, *D.melanogaster* ovary development and embryogenesis are unique model systems for studying the mechanisms underlying intracellular mRNA accumulation. In fact, recent studies show that up to three quarters of probed mRNAs are restricted to specific sites in the cells of early embryos, suggesting that mRNA localisation might be more wide-spread that previously assumed (Lecuyer et al. 2007).

1.3.*oskar* mRNA regulation in the early development of *D.melanogaster*

1.3.1. Biological function of Oskar

The *oskar* gene was cloned in 1991 and the mRNA shown to localise at the posterior pole of the oocyte and the embryo (Ephrussi et al. 1991; Kim-Ha et al. 1991).

Flies with lowered Oskar protein activity develop normally into adulthood, but viable females do not produce any offspring ("grandchildless phenotype"). This is due to a lack of ovaries that develop from the embryonic pole cells (Lehmann and Frohnhofer 1989). Embryos from *oskar* alleles that express no Oskar protein arrest development. The embryos do not form pole cells and also do not develop an abdomen ("posterior group phenotype"; Figure 3A). Conversely when Oskar is over-expressed, embryos develop with two posterior abdomens and two terminal structures (Ephrussi and Lehmann 1992; Smith et al. 1992). These mirror image embryos show the "bicaudal phenotype" (Figure 3A). Thus local Oskar activity is required for correct abdomen and germ cell formation.

oskar mRNA encodes two protein isoforms, Long and Short Oskar, that are translated from alternative start codons. Both isoforms are simultaneously expressed from stage 9 onwards, when *oskar* mRNA first enriches at the posterior pole, but have different functions (Markussen et al. 1995; Rongo et al. 1995). Short Oskar alone can induce posterior structures and pole cells, while Long Oskar can not stimulate germ cell formation and only poorly induces abdominal structures (Markussen et al. 1995). In *oskar* mutants that expressed Short Oskar protein alone both the protein and the RNA diffuse away from the posterior cortex from stage 10 of oogenesis onwards, suggesting that the main function of Long Oskar might be anchoring of Short Oskar and *oskar* mRNA (Vanzo and Ephrussi 2002). Recently Oskar protein was implicated in mediating asymmetric endocytosis in the egg chamber and it was proposed that this could allow the maintenance of localisation (Vanzo et al. 2007; Tanaka and Nakamura 2008).

Another biological function for *oskar* was found in oocyte development: oogenesis in the absence of any *oskar* message ("oskar RNA null flies") arrests prematurely at stage 7. This block in development occurs before any Oskar protein is translated and it was shown that this early function solely depends on the *oskar* 3'UTR RNA (Jenny and Hachet et al. 2006).

In summary, *oskar* RNA (3'UTR) controls ovary development and Oskar activity is required for abdomen development and in the formation of germ cells. To control these processes

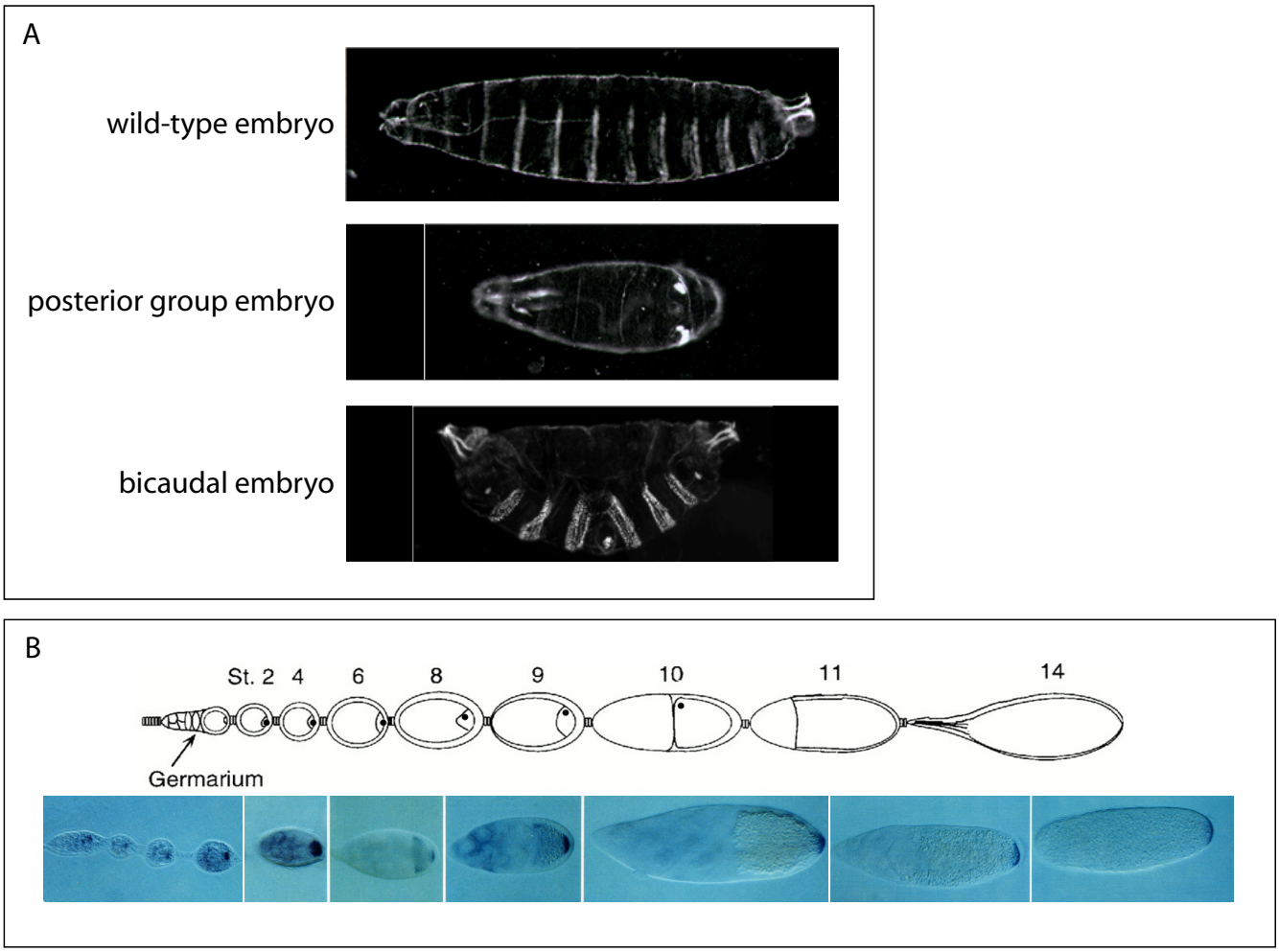


Figure 3: Oskar function and *oskar* mRNA localisation.
A. In wild-type embryos, the head structures (left), the posterior terminal structure, the teson with the filzkörper (right) and the ventral denticle belts are clearly visible. In embryos lacking Oskar activity ("posterior group phenotype") all abdominal segments are missing, clearly noticeable by the lack of denticle belts and a shortened body. In contrast, embryos over-expressing Oskar protein show a mirror image duplication of the posterior half of the body, in the place of the anterior structures ("bicaudal phenotype"). Telsons form at both poles, the posterior abdominal segments are duplicated, and the head structures are absent. B. A detailed diagram of oogenesis stages with the position of the oocyte nucleus indicated (black dot). Beneath, the localisation pattern of *oskar* mRNA at the same developmental stages. At stages 2-7 *oskar* RNA is enriched in the oocyte and is then transiently detected at the anterior pole of stage 8 oocytes. From stage 9 until early embryogenesis, oskar RNA remains restricted to the posterior pole. (Ephrussi et al., 1991).

oskar mRNA must enter the oocyte and become restricted to the posterior pole where local Oskar activity is then established.

1.3.2. *oskar* mRNA localisation

Transcription of *oskar* mRNA occurs in the nurse cells and the mRNA is then transported via the ring canals into the oocyte where it is detectable from stage 2 onwards (Ephrussi et al. 1991; Kim-Ha et al. 1991). At stages 2 to 7, *oskar* mRNA enriches broadly in the posterior half of the oocyte, while during stage 8 the mRNA temporarily associates with the anterior cortex (Figure 3B).

From stage 9 onwards *oskar* mRNA forms a tight crescent at the posterior pole that is maintained until early embryogenesis (Figure 3B; Ephrussi et al. 1991; Kim-Ha et al. 1991). Early reports suggested that the *oskar* 3'UTR contains all *cis*-regulatory signals for posterior localisation, as a chimeric *lacZ-oskar* 3'UTR RNA can localise in wild-type flies at the posterior pole of oocytes, while an RNA lacking the 3'UTR (*lacZ-oskarΔ3'UTR*) cannot (Kim-Ha et al. 1993). It was also reported that localisation elements of other mRNAs reside in the respective 3'UTRs, suggesting that the 3'UTR generally encompasses mRNA localisation signals (Jansen 2001). However Hachet and Ephrussi in 2004 showed that for posterior accumulation in *oskar* RNA null flies, splicing at the intron position 1 together with an intact 3'UTR was necessary for posterior enrichment of *oskar* (Hachet and Ephrussi 2004). In addition, the core components of the exon junction complex show *oskar* mRNA localisation defects at stage 9, while localisation of other mRNAs was unaffected (Newmark and Boswell 1994; Hachet and Ephrussi 2001; van Eeden et al. 2001; Palacios et al. 2004).

Hachet and Ephrussi further showed that posterior accumulation of the *lacZ-oskar* 3'UTR was abolished in *oskar* RNA null flies and that the chimeric 3'UTR could reach the posterior pole, as reported by Kim-Ha et al, but only in the presence of localisable *oskar* mRNA. Thus, they proposed that the *oskar* 3'UTR, by an indirect mechanism, hitch-hikes to the posterior pole of the oocyte.

In contrast, early localisation of the *oskar* mRNA, at stages 2-7 and at stage 8, does not require splicing. Both in egg chambers from wild-type and also from *oskar* RNA null flies, *oskar* 3'UTR RNA alone can enrich in the oocyte, indicating that the *oskar* 3'UTR does contain all *cis*-regulatory sequences required for this early accumulation (Kim-Ha et al. 1993; Hachet and Ephrussi 2004; Wagner et al. 2004; Jenny and Hachet et al. 2006). Although the patterns of localisation of the maternal determinants, *bicoid, gurken* and *oskar* mRNAs at mid

and late oogenesis are clearly distinct, all enrich similarly in the posterior half of the oocyte at stages 2-7 and at the anterior cortex at stage 8 (Berleth et al. 1988; Ephrussi et al. 1991; Kim-Ha et al. 1991; Neuman-Silberberg and Schüpbach 1993). Furthermore, several other mRNAs enrich in the young oocytes and localise similarly to *oskar* mRNA at these stages, for example *fs(K10)* and *BicD* (Figure 2A; Suter et al. 1989; Cheung et al. 1992). Broad regions involved in stage 2-8 oocyte localisation have been characterised, for example in *gurken* mRNA (Van De Bor et al. 2005), but precise mapping was only done for *fs(K10*) mRNA, in which a 44-nucleotide region was shown to fold into an AU-rich stem-loop and to direct localisation (Serano and Cohen 1995; Serano et al. 1995).

In Figure 3B, the stages of *D.melanogaster* oogenesis are summarised as defined by King (1970) and Mahowald and Kambysellis (1980).

1.3.3. Hitch-hiking of *oskar* mRNA

The *oskar* 3'UTR is by itself localisation incompetent but accumulates at the posterior pole in the presence of endogenous, localisable *oskar* mRNA (Kim-Ha et al. 1993; Hachet and Ephrussi 2004). This indirect, hitch-hiking based mechanism of localisation was proposed to depend on either direct on indirect interactions of *oskar* molecules in the oocyte (Hachet and Ephrussi 2004).

Possibly relevant to this, Dr. Christine Brunel observed that the full-length *oskar* 3'UTR can dimerise *in vitro*. This association was maintained if only the 3' half of the 3'UTR, nucleotides 616 to 1151, was included (Figure 4A). This dimerisation-competent fragment encompasses a domain of particularly high conservation between *D.melanogaster* and *D.virilis*, and that was predicted to fold into two stem-loops (SLI and II). The terminal loop of SLII is formed by a GC-rich palindromic sequence, an RNA motif that often is involved in direct RNA-RNA interaction (Brunel et al. 2002). Dr. Brunel experimentally validated this predicted secondary structure by enzymatic and chemical probing (Figure 4B). To determine which nucleotides promote dimerisation, she tested the K_D of the full-length wild-type *oskar* 3'UTR and the consequences of mutations in the SLII domain. Deletions and substitutions within SLII strongly reduced dimerisation (*oskar* 3'UTR: K_D 90nM, *oskar* 3'UTR mutated: K_D 390-590nM). Furthermore, dimerisation could be restored when RNAs containing compensatory mutations *in trans* were analysed, revealing that dimerisation indeed involves the nucleotides of the terminal loop of SLII (Figure 5). In summary, Dr. Brunel showed that

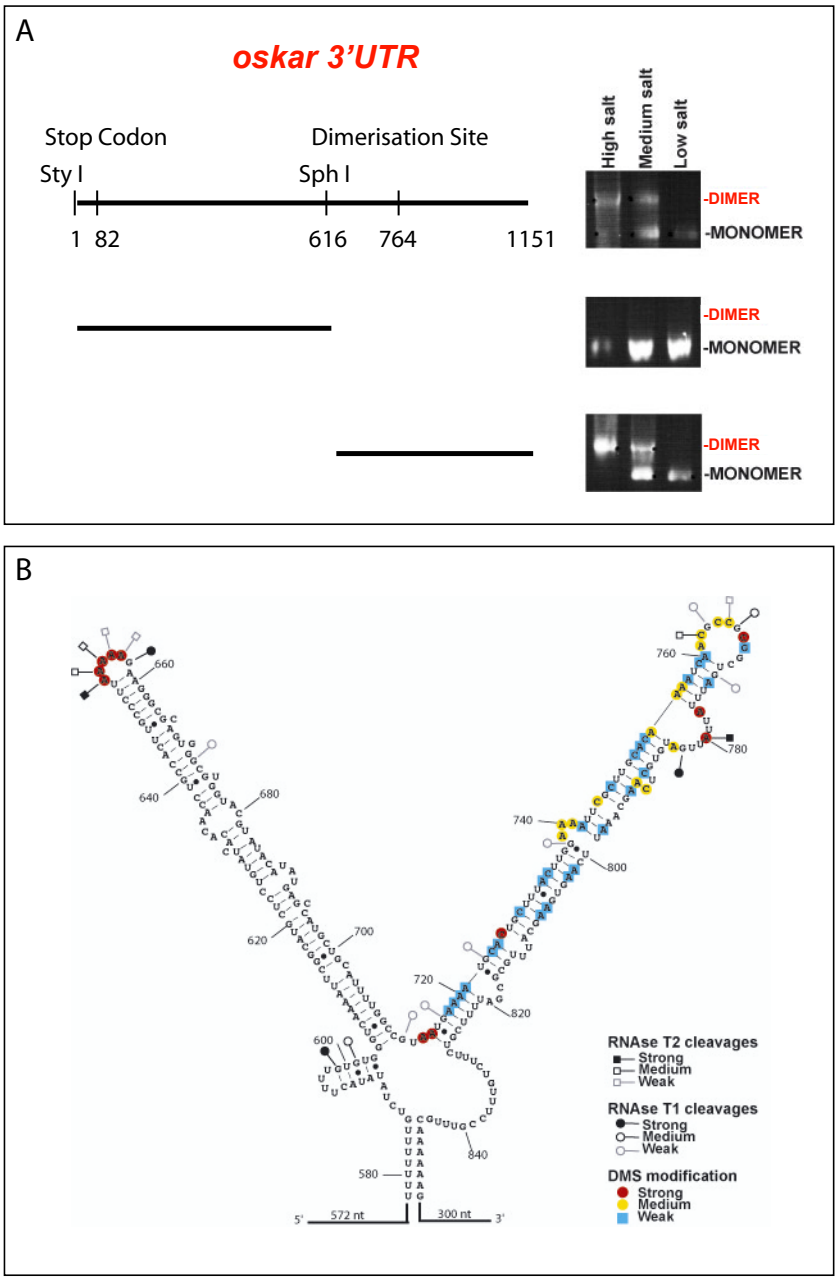


Figure 4: The *oskar* 3'UTR dimerises in vitro via a conserved secondary structure. A. in vitro assay showing dimerisation of full-length *oskar* 3'UTR RNA. RNA dimerisation capacity is restricted to the 3' half of the *oskar* 3'UTR. B. Structure of a highly conserved region within the dimerisation-competent portion of the *oskar* 3'UTR. Chemical and enzymatic probing revealed that this regions folds into two stem-loops. Chemical probing was carried out using dimethyl-sulfate (DMS), which, in RNA, mainly modifies unpaired adenines. For enzymatic probing RNAse T1 and RNAse T2 were used. RNAse T1 efficiently cleaves unpaired guanines, while RNA T2 mostly targets single-stranded RNA at the adenines. (Dr. Christine Brunel, unpublished)

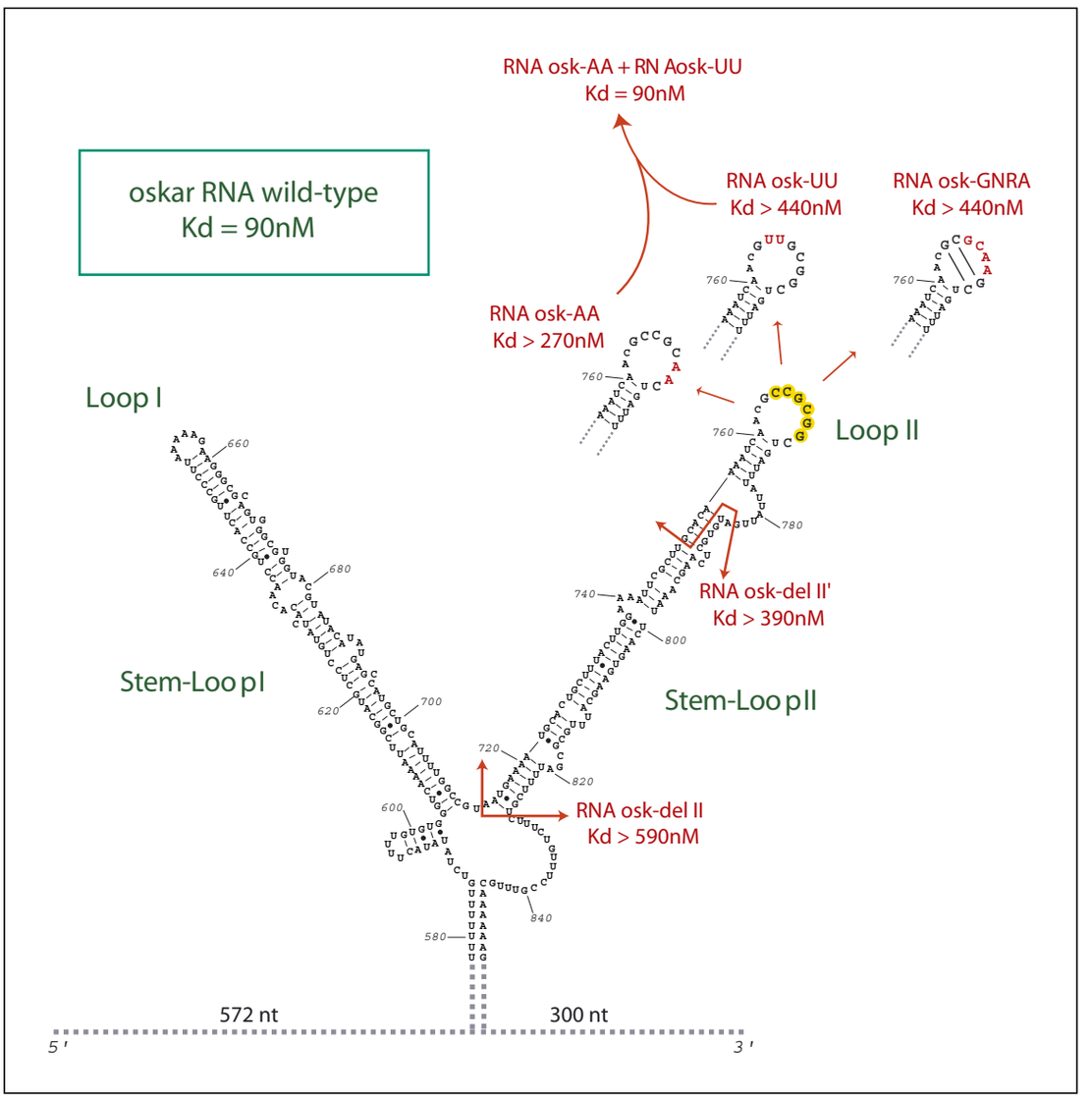


Figure 5: *in vitro* **dimerisation of the** *oskar* **3'UTR depends the palindromic loop of SLII.**
When either stem-loop II was partially deleted (*osk* del-II and del-II') or point mutations introduced in the terminal loop of SLII (*osk*-UU, -AA, -GNRA), dimerisation was strongly reduced. Dimerisation was restored when RNAs bearing compensatory mutations (*osk*-UU and *osk*-AA) were combined. (Dr. Christine Brunel, unpublished)

the *oskar* 3'UTR dimerises *in vitro* via a classical loop-loop interaction involving palindromic nucleotides in the distal loop of Stem-Loop II.

Dimerisation of another RNA, the anterior determinant *bicoid,* has been demonstrated *in vitro* and was shown to occur via a stem-loop in the 3'UTR (Ferrandon et al. 1997; Wagner et al. 2001). The functional relevance of this RNA-RNA interaction *in vivo* in unclear, but injected *bicoid* RNA in the embryo could only assemble into particles with Staufen protein when this dimerisation domain was intact.

1.3.4. Factors involved in *oskar* mRNA localisation

The early localisation of *oskar* mRNA has been shown to depend on an intact microtubule network and similarly, early localisation of *BicD, bicoid, fs(K10)* and *orb* is disrupted when egg chambers are treated with the microtubule depolymerising drug colchicine (Pokrywka and Stephenson 1995). Proteins that are part of the dynein complex, for example BicD, are also necessary for proper *oskar* mRNA localisation during oogenesis (Swan and Suter 1996). At stages 2-7 of oogenesis the microtubules have their minus ends in the oocyte and extend via the ring canals into the nurse cells. In contrast, at stage 8 the microtubule minus-ends are enriched at the anterior pole of the oocyte and the plus ends are focused at the posterior pole (Theurkauf et al. 1992; Clark et al. 1997). At stages 2-8 the mRNAs described above, including *oskar* mRNA, co-localise with the minus-ends of microtubules, supporting the model of a microtubule minus-end directed transport at these stages. Interestingly, apical mRNA localisation in the embryo also involves microtubules and proteins involved in minus-end directed transport (Lall et al. 1999; Wilkie and Davis 2001). It was therefore proposed that mRNA enrichment in young oocytes and apically in embryos could involve the same machinery and the same cis-regulatory sequences (Bullock and Ish-Horowicz 2001).

oskar mRNA transport from the anterior to the posterior pole of the oocyte at stage 9 was also shown to be dependent on active transport. *oskar* mRNA transport requires an intact microtubule network, as *oskar* enrichment at the posterior pole was abolished in flies fed with colchicines (Theurkauf et al. 1992; Clark et al. 1994). Furthermore, a reporter of the plus-ends of microtubules co-localises with *oskar* mRNA at the posterior pole of the oocyte (Clark et al. 1994; Bullock and Ish-Horowicz 2001), and mutants in *kinesin heavy chain* also show mis-localisation of the *oskar* mRNA (Brendza et al. 2000). Thus, while in early oogenesis, at stage 2-8, *oskar* mRNA co-localises with the microtubule minus-ends, from stage 9 onwards the

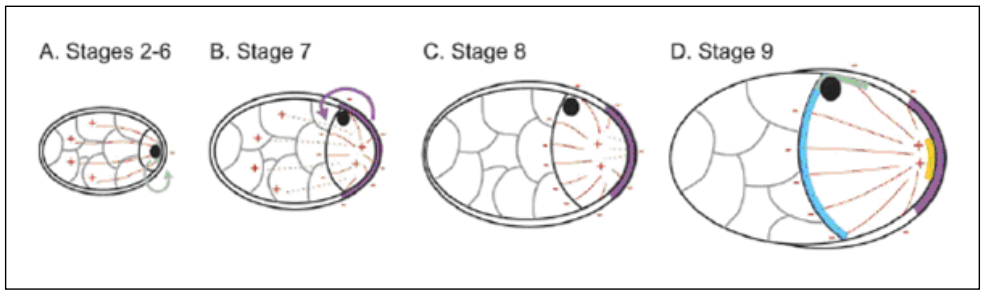


Figure 6: A model of microtubule organisation in *D.melanogaster* **oogenesis.**
In egg chambers of stage 2-7, the microtubule minus ends localised at the posterior of the oocyte and extend their plus-ends anteriorly, towards and into the nurse cells. At stage 8, a re-polarisation of the cytoskeletal organisation occurs, such that the microtubule nucleate along the anterior and lateral cortex, and extend into the volume of the oocyte with a slight enrichment of plus ends focused towards the posterior. (From Steinhauer and Kalderon, 2006)

oskar message associates with the plus-ends instead (Figure 6; Steinhauer and Kalderon 2006).

The proteins mediating *oskar* mRNA localisation have been studied for many years. Mutations in *capu*, *spire* and *staufen* were reported to interrupt posterior patterning activity and, in these mutants, *oskar* mRNA fails to localise at the posterior pole of the oocyte from stage 9 onwards (Schupbach and Wieschaus 1986; Manseau and Schupbach 1989; Ephrussi et al. 1991). Staufen protein was also shown to associate with *oskar* at the anterior and to move with the RNA to the posterior pole of the oocyte (St Johnston et al. 1991). In mutants that disrupt oocyte polarity, Staufen and *oskar* mRNA are both mis-localised to the same ectopic site, and the posterior localisation of Staufen was shown to depend on *oskar* mRNA (Ferrandon et al. 1994; Gonzalez-Reyes et al. 1995; Roth et al. 1995).

Although numerous proteins are implicated in *oskar* transport it is still unclear how *oskar* RNP is linked to the cytoskeleton, but the importance of the microtubules in enriching *oskar* at the posterior pole is clearly evident.

1.3.5. Local expression of Oskar protein

A high local *oskar* activity is critical for embryonic development. For this, Oskar must be enriched at the posterior pole where it is required and depleted from sites where it could cause developmental defects. To restrict Oskar activity, *oskar* mRNA is localised to the posterior pole (Ephrussi et al. 1991; Kim-Ha et al. 1991). In the oocyte, *oskar* message is translated only after it is tightly localised at the posterior pole from stage 9 onwards (Kim-Ha et al. 1995). Thus, silencing of unlocalised *oskar* additionally ensures a local activity of Oskar protein.

Translational repression crucially depends on the protein Bruno (encoded by the *aret* gene), which co-localises with *oskar* mRNA in the oocyte (Webster et al. 1997). Bruno can directly bind to five Bruno-response elements (BRE) located in the *oskar* 3'UTR. When an *oskar* transgene bearing mutated BREs is expressed, the mRNA localises normally but Oskar protein is prematurely expressed resulting in bicaudal embryos (Kim-Ha et al. 1995; Webster et al. 1997). Other proteins were implicated in translation control of *oskar*. For example the Dhh1/RCK homologue of the fly, Me31B, co-localises with *oskar* mRNA and, when mutated, causes oogenesis arrest and a premature expression of Oskar in the germline (Nakamura et al. 2001). Dhh1 was shown in yeast cells to be an integral part of P-bodies, structures that form for example under stress conditions and control mRNA stability and storage (Parker and

Sheth 2007). Regulated activity of PKA is also required to restrict *oskar* protein expression to the posterior of the oocyte (Yoshida et al. 2004). However, apart from Bruno these proteins have so far not been reported to directly bind *oskar* mRNA and in addition they are also involved in progression of early oogenesis. Their effect on *oskar* mRNA translation might therefore not necessarily be direct. Thus the only *cis*-regulatory sequences involved in translational repression of the *oskar* message remain the BREs.

At stage 9, upon *oskar* mRNA localisation at the posterior pole of the oocyte, Oskar protein becomes detectable at this site. How exactly Oskar translation is activated is poorly understood. Some mutations in Staufen protein that do not affect *oskar* mRNA localisation, nevertheless abrogate Oskar protein production (Micklem et al. 2000). Aubergine, closely related with Piwi, Ago1 and Ago2, was also reported to have a role in Oskar translation, but which role exactly Aubergine plays remains elusive (Harris and Macdonald 2001). In ovaries mutant for *orb*, the *D.melanogaster* CPEB (Cytoplasmic Polyadenylation Element Binding protein) homologue of the fly, the length of the *oskar* poly(A) tail is shortened and the amount of Oskar protein at the posterior pole is reduced (Markussen et al. 1995; Chang et al. 1999; Castagnetti and Ephrussi 2003). Additionally a cytoplasmic activity of the nuclear poly(A) binding protein, PABP2 regulates the length of *oskar* poly(A) tail. Together with the deadenylase CCR4, PABP2 can shorten poly(A) tails of target mRNAs (Benoit et al. 2005), a finding that highlights the importance of this type of regulation for *oskar* mRNA translational control.

Cis-regulatory sequences necessary for translational activation of *oskar* mRNA are thus the polyadenylation signal in the 3'UTR and additionally a 414-nucleotide region at the 5' end of the *oskar* ORF. This 414-nucleotide sequence is required to initiate posterior translation of *oskar* mRNA (Gunkel et al. 1998). In *oskar* mRNA with mutated Bruno binding sites, translation is initiated in the complete oocyte (Kim-Ha et al. 1995), suggesting that this activator element might be required to circumvent Bruno mediated repression at the posterior.

1.3.6. RNP complexes

Localised mRNAs in several systems have been observed in granular aggregates and it is believed that for each step of mRNA regulation new proteins associate, while other proteins disassociate from these ribo-nucleoprotein particles (RNPs; e.g. (Ainger et al. 1993). Thus during its maturation (processing, nuclear export, transport, anchoring and translation) an RNP must be heavily remodelled. Such remodelling is thought to be dependent on RNA

helicases as they are often integral parts of these particles but also on post-transcriptional modifications of the involved proteins and on oligomerisation (Lewis and Mowry 2007). For example the integrity of the myelin basic protein (MBP) RNA containing granules oligodendrocytes depends on HnRNP A2, a protein that can dimerise and is thought to bind to multiple sites in the RNA (Carson et al. 2001).

For correct localisation and translation of *oskar* mRNA, as described above, the activity of numerous factors is necessary. Although some proteins affect *oskar* indirectly, a large number of them must still associate and possibly directly bind to the RNA such as Bruno, Hrp48 and PTB (Webster et al. 1997; Yano et al. 2004; Besse and Lopez de Quinto et al., submitted). Further biochemical data also showed that the *oskar* 3'UTR RNA is found in heavy particles (Wilhelm et al. 2003; Chekulaeva et al. 2006). These heavy particles are known to contain large quantities of both protein and RNA and it is estimated that *oskar* particles include around 100 molecules of *oskar* RNA (Glotzer et al. 1997). It is currently unclear what *cis*-regulatory signals are responsible for assembly and remodelling of the *oskar* mRNP.

1.4. Aim of the thesis

Part I: A novel mechanism for translational repression of the *D.melanogaster* germ line determinant *oskar* based on dimerisation of RNA molecules

It was observed that *oskar* 3'UTR RNA molecules can indirectly accumulate at the posterior pole of the oocyte when localisable *oskar* mRNA is present in *trans* (hitch-hiking). Further it was shown that the *oskar* 3'UTR RNA can dimerise *in vitro* via a dimerisation domain. This led us to hypothesise that the indirect localisation of the *oskar* 3'UTR RNA to the posterior pole might be based on a direct RNA-RNA interaction of *oskar* molecules.

I first investigated the relevance of the dimerisation domain for hitch-hiking *in vivo*. To this end I expressed *oskar* 3'UTR RNAs with and without mutations in the dimerisation domain in the ovary and probed the ability of these RNAs to enrich at the posterior pole by hitch-hiking. For this purpose, I generated transgenic flies and expressed the transgenes in the presence of endogenous, localisable *oskar* mRNA.

Secondly, I analysed the relevance of the dimerisation domain for regulation of *oskar* mRNA. I generated a genomic *oskar* mRNA bearing a 2-nucleotide substitution in the dimerisation domain that *in vitro* abrogates RNA dimerisation. I expressed this transgenic *oskar* mRNA in *oskar* RNA null flies and analysed whether this mutated *oskar* mRNA could substitute for wild-type *oskar* mRNA in the fly development.

Part II: Characterisation of the oocyte entry signal in the *oskar* 3'UTR RNA

oskar 3'UTR RNA enriches in young egg chambers and its distribution is highly similar to that of other localised mRNAs. It was therefore interesting to determine to what extent the cis-regulatory sequences involved in localisation of these RNAs are also similar.

The purpose of my analysis was therefore, to define the oocyte-localisation signal in the *oskar* 3'UTR.

I started by mapping the region of the *oskar* 3'UTR sufficient for early oocyte localisation. For this purpose I designed deletions in the *oskar* 3'UTR that do not interrupt highly conserved 3'UTR regions. Transgenes bearing these *oskar* 3'UTR fragments were then expressed in young egg chambers and their mRNA localisation analysed by *in situ* hybridisation.

Having defined a minimal region that supports oocyte localisation , I tested exactly what features of this element were required for correct mRNA localisation. To this end, I analysed the localisation of mRNAs in which the secondary structure of the RNA region mediating early localisation was disrupted.

Finally, to try to identify a common motif that could direct microtubule minus-end localisation of RNAs in the *D.melanogaster* oocyte and embryo, I compared the *oskar* oocyte localisation element to the functionally equivalent signals in *gurken* and *fs(K10)* RNAs, and also to the apical localisation elements in *wingless*, *hairy* and *fushi-tarazu*.

2. Materials and Methods

2.1.*D.melanogaster* genetics

2.1.1. Fly husbandry

Flies were maintained according to standard procedures (Ashburner, 1989) and grown on standard corn meal molasses agar. Fly stocks were generally stored at 18°C. Unless stated otherwise the crosses and experiments were carried out at 25°C. Prior to dissection flies were kept for either 1 day on yeast at 25°C or for 2 days at 21°C.

2.1.2. Stocks

For this study we made use of the following fly stocks: w^{1118}, reference stock used as wild-type control, osk^{A87} (Vanzo and Ephrussi 2002), $Df(3R)^{pXT103}$ (Lehmann and Nusslein-Volhard 1986). In combination osk^{A87} and $Df(3R)^{pXT103}$ give rise to *oskar* RNA null flies (Jenny, Hachet et al. 2006), *pCog*-Gal4:VP16 (Rørth 1998), *nanos*-Gal4:VP16 (Rørth 1998), *maternal-α-tubulin*-Gal4:VP16 (Hacker and Perrimon 1998).

2.1.3. Generation of transgenic flies

Transgenic flies were generated by P-element transformation (Rubin and Spradling 1982) using the pUASp vector (Rørth 1998). The pUASp vector was modified by removing the *fs(K10)* 3'UTR-signal. *fs(K10)* mRNA itself is localised during oogenesis and thus could potentially interfere with the localisation of *oskar* 3'UTR reporter RNAs.

Insertion of the pUASp-constructs into the genome of recipient flies is random and the expression of the transgenes is influenced by position effects. For better comparison, the expression levels were quantified by quantitative RT-PCR. This was done for several insertions per transgenic line.

2.1.4. Ectopic expression using the UAS/GAL4 system

In *D.melanogaster,* transgenes can be expressed in a temporally and spatially restricted pattern with the GAL4/UAS system (Brand and Perrimon 1993). In the pUASp vector all transgenes are under the control of 14 UAS promoters and the P-transposase promoter. The expression of the transgene is then inducible, by crossing transgenic flies to a fly expressing the Gal4 under a germ line specific promoter.

To analyse *oskar* RNAs in the presence of endogenous *oskar* mRNA, expression of the transgenes was induced by Gal4 under the control of the *maternal-α tubulin* promoter. The results were confirmed by alternatively inducing expression under the control of the combined *pCog* and *nanos* promoters (data not shown). These promoters allow a strong and continuous expression of the transgenes during oogenesis. In osk^{A87}/ $Df(3R)^{pXT103}$ flies all transgenes were expressed using both the *pCog*-Gal4:VP16 and *nanos*-Gal4:VP16 promoters.

2.2.Molecular Biology

2.2.1. Constructing transgenes

Generating *oskar* fragments

pUC119-T7 containing *oskar*-3'UTR-WT, *oskar*-3'UTR-GNRA, *oskar*-3'UTR-UU, *oskar*-3'UTR-AA and *oskar*-3'UTR-ΔSLIId (="ΔOL") were gifts from Dr. Christine Brunel. *oskar* 3'UTR-WT, -GNRA and–UU were amplified by PCR from the respective pUC119-T7 DNA template using primers HJ20 and HJ19. The *oskar* 3'UTRs were then subcloned to TOPO pCR II (Invitrogen). After BamHI digestion of the pCR II, the inserts were cloned to pUASpGWΔK10.

oskar-3'UTR-SLI-II was generated by PCR amplification with HJ32 and HJ33 primers. The resulting fragment was subcloned to pCR II and a BamHI digested fragment fused to pUASpGWΔK10.

The *oskar* 3'UTR deletions were PCR amplified from pUC119-T7 *oskar* 3'UTR-WT template using the appropriate primers, subcloned to pCR II and BamHI fragments fused to pUASpΔK10GFP. *oskar* 3'UTR-region1+2 was amplified using HJ20 and HJ33; *oskar* 3'UTR-region2+3 was amplified using HJ32 and HJ19; *oskar* 3'UTR-region1 was amplified

using HJ20 and HJ51; *oskar* 3'UTR-region2 is equal to the *oskar* 3'UTR-SLI-II (see above); *oskar* 3'UTR-region3 was amplified using HJ50 and HJ19; *oskar* 3'UTR-region2b+3 was amplified using HJ39 and HJ19; *oskar* 3'UTR-region2b was amplified using HJ39 and HJ33.
To generate mutated versions of *oskar* 3'UTR-region2 (see section 5.1.2), mutagenesis PCR using pCR II-SLI-II (region2) as template was performed. The mutagenised SLI-II was then BamHI digested and cloned to pUASpGWΔK10.

Genomic *oskar-AA* was generated performing mutagenesis PCR using pSP72-*osk*WT as template (Hachet and Ephrussi 2004). *oskar*-AA was subsequently PCR amplified using SGBamHIfwd and SG3'UTRrvs primers (designed by Sanjay Ghosh) and subcloned to pCR II. A BamHI fragment was then subcloned to pUASpΔK10.

Generating pUASpΔK10

To generate pUASpΔK10 the K10 3'UTR was removed by XbaI and PstI digestion, the ends blunted and the vector religated.

To generate pUASpGWΔK10 the GFP-ORF was amplified using primers attaching KpnI and NotI sites (HJ26, HJ27) from a pPGW (Carnegie Institution of Washington) template DNA. This fragment was subcloned to pCR II, a KpnI/NotI fragment isolated and fused to KpnI/NotI digested pUASpΔK10.
To receive *oskar* 3'UTR fragments the pUASpΔK10 and pUASpGWΔK10 were BamHI digested and dephosphorylated.

2.2.2. Cloning of *oskar* 3'UTR orthologs

To clone the *oskar* 3'UTRs from *D.yakuba, D.pseudoobscura, D.immigrans, D.mercantorum* and *Zaprionus sepsoides* (Gift from Ruth Lehman) total RNA from oocytes was isolated using Trizol (Invitrogen) and polyadenylated RNA was enriched (DynaBeads mRNA purification kit). The cDNA was generated (Invitrogen Superscript First Strand Synthesis kit) and the DNA amplified using a primer designed to bind a conserved region in the *oskar* coding sequence (HJ9) and an Oligo-d(T) primer. The amplified product was cloned into pCR II TOPO vector (Invitrogen) and sequenced.

2.2.3. Mutagenesis PCR

For mutagenesis the PCR QuikChange® XL Site-Directed Mutagenesis Kit from Stratagene was used according the suppliers instructions.

oskar-AA was mutagenised by PCR using HJ110 and HJ111 primers and pSP72-oskWT template DNA.

The region-2 mutants used in section 5.2 were generated using specific primers and TOPO pCR II containing region2 (pCR II-SLI-II) as template DNA. To generate *2-5'3'mut* pCRII-2-5'mut was used as template DNA. The following primers were used: *oskar* 3'UTR *2-5'mut* (HJ76, HJ77); *oskar* 3'UTR 2-3'mut (HJ78, HJ79); *oskar* 3'UTR 2-5'3'mut (HJ80, HJ81); *oskar* 3'UTR 2-Δloop (HJ86, HJ87); *oskar* 3'UTR 2-GCstem was mutagenised in two steps: the first PCR (primers HJ84, HJ85) created *oskar* 3'UTR *2-GCstem3'* and the second PCR (HJ82, HJ83) using *oskar* 3'UTR *2-GCstem3'* as template DNA created the fully mutant 2-GCstem.

2.2.4. Primer List

Primer name	Primer sequence
HJ9 deg_UTR1	c g a t t t g a t t g c t T t c a g
HJ19_pUCrevBamH1	TAG AGG ATC CCC TGG TAG GC
HJ20_pUCforBglII	GAT CGA GAT CTC GAT CCC GC
HJ26_egfpKpn1_for	TAT AGG TAC CAT GGT GAG CAA GG
HJ27_egfpNot1_rev	ATA TAG CGG CCG CCT TGT ACA GCT
HJ32_SLI/II_forBamHI	TTGGATCCTGTTCTATATAC
HJ33_SLI/II_revBamHI	TTGGATCCAACTTTTTTGCA
HJ39_SLII_BglII	GCTCGAGATCTAATGAAAATGC
HJ43 for	TGC TGC CCG ACA ACC ACT ACC TG
HJ50_osk3'_for	TTGGATCCTGCAAAAAAGTT
HJ51_osk3'_rev	TTGGATCCGTA TAT AGA ACA
HJ76	GCTTTACTTGGAAAATTCGCTTGCACATTTAGTACGCCGCGGCTG
HJ77	CAG CCG CGG CGT ACT AAA TGT GCA AGC GAA TTT TCC AAG TAA AGC
HJ78	CACAAAATCAACGCCGCGGCACTAAAATTATTGATGTGCTCAAGC
HJ79	GCT TGA GCA CAT CAA TAA TTT TAG TGC CGC GGC GTT GAT TTT GTG
HJ80	CGCTTGCACATTTAGTACGCCGCGGCACTAAAATTATTGATGTGC
HJ81	GCA CAT CAA TAA TTT TAG TGC CGC GGC GTA CTA AAT GTG CAA GCG
HJ82	CGCTTGCACAGGGCTGACGCCGCGGCCAGCCCATTATTGATGTGC
HJ83	GCA CAT CAA TAA TGG GCT GGC CGC GGC GTC AGC CCT GTG CAA GCG
HJ84	CACAAAATCAACGCCGCGGCCAGCCCATTATTGATGTGCTCAAGC
HJ85	GCT TGA GCA CAT CAA TAA TGG GCT GGC CGC GGC GTT GAT TTT GTG
HJ86	CGC TTG CAC AAA ATC AAC GCT GAT TTA TTA TTG ATG TGC
HJ87	GCA CAT CAA TAA TAA ATC AGC GTT GAT TTT GTG CAA GCG
HJ88	CGA CTC ACT ATA GGG TCG ACG GTA CCA ATT TCT ATT AAA GGT TCC

HJ89	GGA ACC TTT AAT AGA AAT TGG TAC CGT CGA CCC TAT AGT GAG TCG
rp49F	GCTAAGCTGTCGCACAAA
rp49R	TCCGGTGGGCAGCATGTG
HJ110	CGC TTG CAC AAA ATC AAC GCC GCAACT GAT TTA TTA TTG ATG TGC
HJ111	GCA CAT CAA TAA TAA ATC AGT TGC GGC GTT GAT TTT GTG CAA GCG
SGBamHIfwd	CCGGGGATCCAAGAATATTGGATCAC
SG3'UTRrvs	GAACATAGCTTAGAGCAAACAAAATCATTG

2.2.5. RNA isolation

5-10 ovaries from adult flies were dissected and dissociated in 200 µl of Trizol (Invitrogen). Then 600 µl of Trizol were added and the RNA isolated according to the manufacturers instructions.

2.2.6. cDNA synthesis

cDNA was synthesised using ThermoScript RT-PCR System (Invitrogen) and following the manufacturer's instructions. For each experiment the same amount of RNA (typically 2-3µg was used).

2.2.7. quantitative RT-PCR

Quantitative RT-PCR was performed on cDNA generated from ovaries of flies expressing the respective transgenes. Amplified product was detected using the SYBRGreen (Ambion) system on an ABIPrism7500 real time PCR apparatus. *GFP* was amplified using primers specific for the GFP ORF (HJ27/HJ43) and normalized to *rp49* (amplified using the primers rp49F, rp49R). The amplification efficiency was determined using serial dilutions of a cDNA mix from all samples and was not allowed to go below 70%. In each experiment three replicates were analysed and the experiment repeated two to three times. As the negative control w^{1118} flies not expressing the transgenic *oskar* RNA were included. For the interpretation of the results the Relative Expression Software Tool-Mutiple Condition Solver version 1 (REST-MCS) was used (Pfaffl 2001).

2.2.8. *in vitro* transcription for *in situ* probe generation

The *gfp* antisense probe was generated by *in vitro* transcription with T7 polymerase (Ambion, Megascript) from a pCRII-TOPO plasmid containing the GFP-ORF (pCR II-GFP). 10µg of

plasmid DNA was KpnI linearised and purified using Phenol-Chloroform extraction. The probe showed no signal on negative control ovaries from w^{1118} flies.

2.3. Western blotting

Protein levels were analysed by 8.5% SDS-PAGE followed by western blotting according to standard procedures (Markussen, et al. 1995).

Proteins were detected using rabbit anti-Kinesin heavy chain (1:30000 Cytoskeleton) and rabbit anti-Oskar (1:2000) antibodies and HRP-conjugated secondary antibodies (GE Healthcare). Detection was done using enhanced chemiluminescence reagent by mixing equal volumes of the Enhanced Luminol Reagent and the Oxidizing Reagent (NEL105, PerkinElmer). The membrane was then exposed to Kodak X-OMAT MR Film for 10 seconds to 5 minutes.

Extracts were obtained by manually disintegrating 10 pairs of ovaries or 20 embryos in 100µl 2x SDS–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer. The Samples were then boiled at 95°C for 5 minutes and 1-4µl of extract was loaded per lane.

2.4. Ovary staining procedures

2.4.1. Immunofluorescence staining of Drosophila egg chambers

Fluorescent antibody staining was performed as previously described (Tomancak et al. 2000). Antigens were detected by rabbit anti-Staufen (1:1500) and rat anti-Oskar (1:3000; unpublished) primary antibodies. For detection secondary antibodies coupled to the following dyes were used at a 1:500 dilution: Cy5 (Jackson Immuno Research Laboratories) and AlexaFluor488 (Invitrogen).

2.4.2. Fluorescent *in situ* hybridisation

In situ hybridisation was performed as described elsewhere (Hachet and Ephrussi 2004).

2.4.3. FISH/immunofluorescence double labelling of ovaries

Immunostaining coupled with *in situ* hybridisation was carried out as previously described (Vanzo and Ephrussi 2002).

2.5. Determination of hatching rates

Transgenic *oskar-WT*, *oskar*-AA, *oskar*-AA/*gfp-oskar* 3'UTR-WT and *oskar*-AA/*gfp-oskar* 3'UTR-UU mRNAs were expressed in osk^{A87}/Df(3R)p^{XT103} flies. Hatching was assayed essentially as describe previously (Coutelis and Ephrussi 2007). Flies were allowed to lay eggs for up to 12 hours and several male w^{1118} flies were added per vial. As control, w^{1118} female flies were also analysed.

2.6. Analyses of embryonic phenotypes

For cuticle preparations, embryos were collected on apple-plates with fresh yeast. After 24 hours, the hatched larvae were removed together with the yeast. The unhatched embryos were de-chorionated in 25% sodium hypochloride for 2 minutes, collected in PBS (0.1%Triton-X), washed twice in 100% methanol, mounted in Hoyer's medium and dried over-night at 65°C (Nüsslein-Volhard, 1984).

2.7. Determining *oskar* reporter RNA Hitch-hiking in egg chambers

To test for hitch-hiking of *gfp-oskar* 3'UTR reporters along with endogenous *oskar* mRNA the transgenes were expressed in the germ line under the control of *maternal-α-tubulin*-Gal4. For analysing hitch-hiking of *gfp-oskar* 3'UTR-WT/-UU reporters along with transgenic *oskar*-AA mRNA, both transgenes were co-expressed in oskar RNA null flies under the control of *pCog* and *nanos* Gal4.

For each experiment it was monitored that the *gfp-oskar* 3'UTR reporter RNAs were expressed to the same levels by qRT-PCR. Hitch-hiking was then analysed by scoring posterior pole enrichment of the *gfp* RNA in the oocyte.

oskar-AA does not enrich fully at the posterior pole of stage 9 egg chambers. The *oskar* RNA null egg chambers expressing both *oskar*-AA and *gfp-oskar* 3'UTR reporters were therefore co-stained for Staufen protein and *gfp* RNA. Only egg chambers that showed posterior Staufen enrichment were then used to analyse hitch-hiking.

2.8. Software/Websites

The genomic sequences of fly species are available at: http://genome.ucsc.edu/

Sequences were aligned using ClustalW at: http://www.ebi.ac.uk/clustalw/index.htm (Larkin et al. 2007)

Alignments were visualised using http://ani.embl.de/alignment/ (Laurence Ettwiller) that includes Blastz and Jalview.

RNA sequences were folded using http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1-2.3.cgi (Zuker 2003) or http://rna.tbi.univie.ac.at/cgi-bin/RNAalifold.cgi (Gruber et al. 2008)

2.9.Microscopy

For all analyses a Confocal microscope (Leica) equipped with a scan head (TCS SP2) and an oil-immersion 20x 1.4 NA objective was used. Images were acquired at a 2x zoom and edited with Adobe Photoshop CS.

3. Results Part I

A novel mechanism for translational repression of the *D.melanogaster* germ line determinant *oskar* based on dimerisation of RNA molecules.

3.1. The *oskar* RNA dimerisation domain is widely conserved among *Drosophilidae* species

3.1.1. *oskar* 3'UTR from *D.pseudoobscura, D.yakuba, D.mercantorum, D.immigrans, D.virilis* and *Zaprionus sepsoides*

Conservation of primary sequence and secondary structure often points to functionally important regions in mRNAs. Before analysing the relevance of the dimerisation domain *in vivo*, the sequence similarities between the 3'UTRs of *oskar* orthologues were compared. The *D.virilis oskar* (*virosk*) was previously cloned and *virosk* shown to localise at the posterior pole of the *D.virilis* oocyte (Webster et al. 1994). The genome sequences of *D.pseudoobscura* and *D.yakuba* were also available from public databases[2]. To obtain sequences from additional species for a more informative alignment, I cloned the *oskar* 3'UTR orthologues from *D.yakuba, D.pseudoobscura, D.immigrans, D.mercantorum* and *Zaprionus sepsoides* using an oligo-dT primer and a primer specific for the conserved 3' end of the coding region. The alignment of the *oskar* 3'UTR sequences from all available species shown in

Figure 7 revealed several regions of high similarity and, among these, the region containing the dimerisation domain. Six nucleotides that form a palindromic sequence have been shown to be critical for dimerisation *in vitro*. These six nucleotides were entirely conserved across species (

Figure 7, highlighted in purple). Furthermore, the orthologous *oskar* RNAs were also found to be localised at the posterior pole of oocytes of the respective species as shown by *in situ* hybridisation with a probe specific for the orthologous *oskar* mRNA, suggesting that *oskar* RNA localisation is conserved (Appendix I).

The secondary structure of the different *oskar* 3'UTRs was predicted using the *mfold* algorithm version 2.3 (Zuker 2003). This version allows variation of the folding temperature which is restricted to 37°C in later versions. The *oskar* 3'UTR secondary structure was folded at 25°C, as flies are usually raised at this temperature in the laboratory. The part of *oskar* 3'UTR including the dimerisation domain was predicted by *mfold* to fold into a strikingly similar structure in all species, with the six-nucleotide palindromic sequence always forming a terminal loop (Figure 8, shown are *D.melanogaster*, *D.yakuba*, *D.pseudoobscura* and

[2] For example UCSC genome browser.

D.virilis). For comparison, we also used the RNAalifold programme that predicts a consensus secondary structure based on a set of aligned sequences (Gruber et al., 2008). The RNAalifold predicted the aligned *oskar* 3'UTRs to adopt the stem-loop structure in the region of the *D.melanogster* dimerisation domain also predicted by *mfold* (data not shown).

The primary sequences and predicted secondary structures of *D.yakuba, D.pseudoobscura, D.virilis, D.mercantorum* and *D.immigrans oskar* 3'UTRs were compared in detail with the sequence and structure of *D.melanogaster oskar* 3'UTR (Figure 9; Appendix II). Most of the mismatches in the primary sequence of the dimerisation domain are either in predicted loops or are semi-compensatory (e.g. changing a G-C to an G-U pair) or compensatory (e.g. changing a G-C to a A-T pair). Mutations in loops do not affect the secondary structure and semi-compensatory and compensatory mutations maintain the secondary structure. Only two nucleotides differ in the entire dimerisation domain between of *D.yakuba* and *D.melanogaster* and neither affects the secondary structure. Comparing *D.pseudoobscura* and *D.virilis* to *D.melanogaster,* as expected from the greater evolutionary distance, more nucleotide changes are observed in the dimerisation domain, yet only one nucleotide change in each affects the secondary structure (summarised in Table 1). Thus nearly all of the mismatches on the level of primary sequence nevertheless allow the dimerisation domain to fold into a stem-loop structure. This above average frequency of semi- and fully-compensatory mutations suggests an importance of the secondary structure. These data strongly support the structure model proposed by Christine Brunel for the dimerisation domain (Figure 4B in the introduction).

Table 1: Summary of nucleotide differences in the *oskar* dimerisation domain between several Drosophilids and *D.melanogaster*. Nucleotide changes were categorised into changes that disrupt the secondary structure or changes that maintain this structure. If alterations occur in loops, change a G-C into a G-U pair or a A-T into a G-C pair, they do not affect the secondary structure of the RNA.

***D.melanogaster* versus…**	**Total number of nucleotide changes**	**Mis-matches disrupting the structure**	**Mis-match in loops**	**Semi-compensatory mis-match**	**Compensatory mis-match**
D.yakuba	**2**	**-**	**1**	**1**	**-**
D.pseudoobscura	**12**	**1**	**7**	**4**	**-**
D.immigrans	**52**	**9**	**28**	**5**	**10**
D.virilis	**39**	**1**	**18**	**4**	**16**
D.mercantorum	**43**	**-**	**20**	**5**	**18**

This analysis reveals that the dimerisation domain is highly conserved, both on the level of primary sequence and secondary structure, with the loop of the dimerisation domain being invariant between species. I therefore concluded that this region likely plays a role in the *in vivo* regulation of *oskar* mRNA.

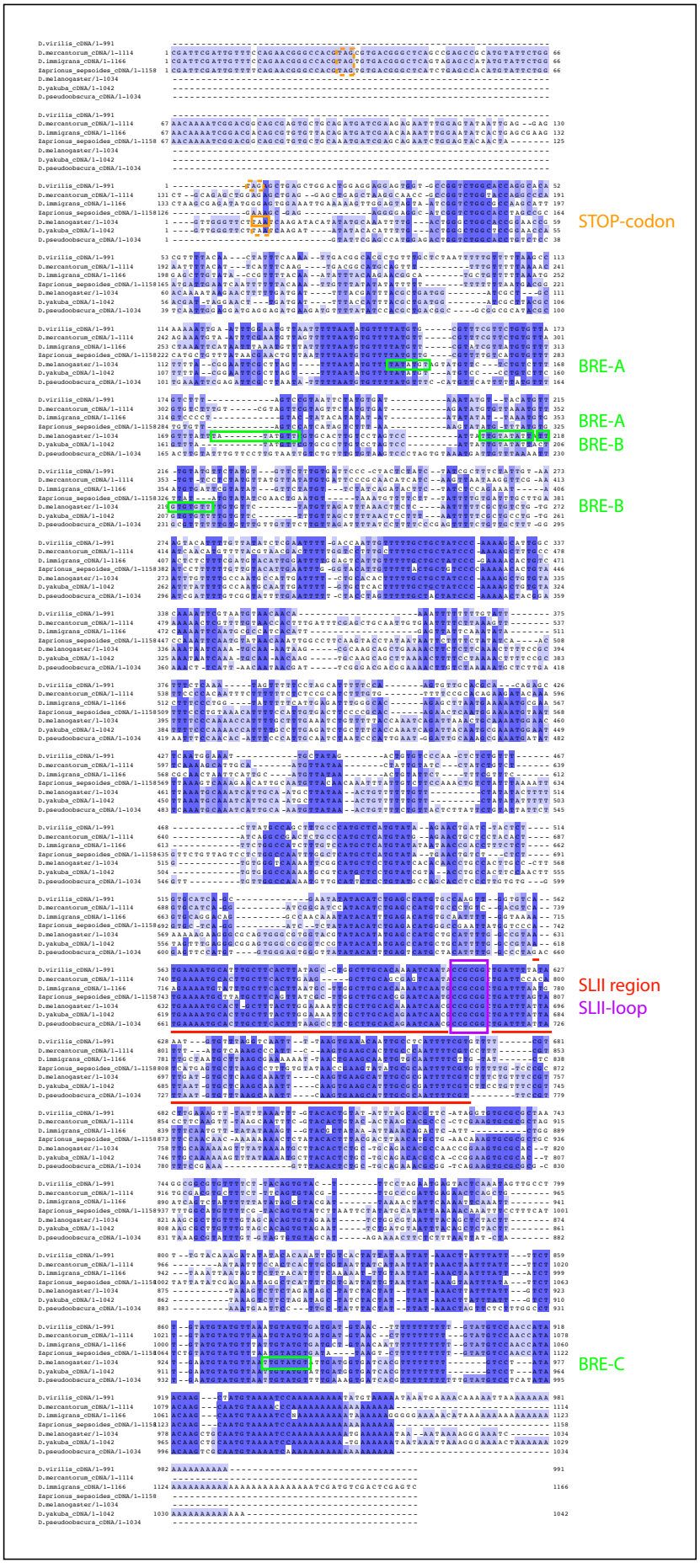


Figure 7: The *oskar* 3'UTR contains regions of high conservation among *Drosophilidae*.
Alignment of *oskar* 3'UTR cDNA sequences from *D.melanogaster, D.yakuba, D.pseudoobscura, D.virilis, D. immigrans*, *D.mercantorum* and *Zaprionus sepsoides*. Shown in blue are regions of similarity: dark blue >85%, blue >71%, light blue >42%. Bruno response elements (BRE) of *D.melanogaster oskar* are highlighted in green. The conserved dimerisation domain folding into a stem loop (SLII, as characterised by Dr Christine Brunel) is underlined in red, with the nucleotides forming the loop highlighted in purple.

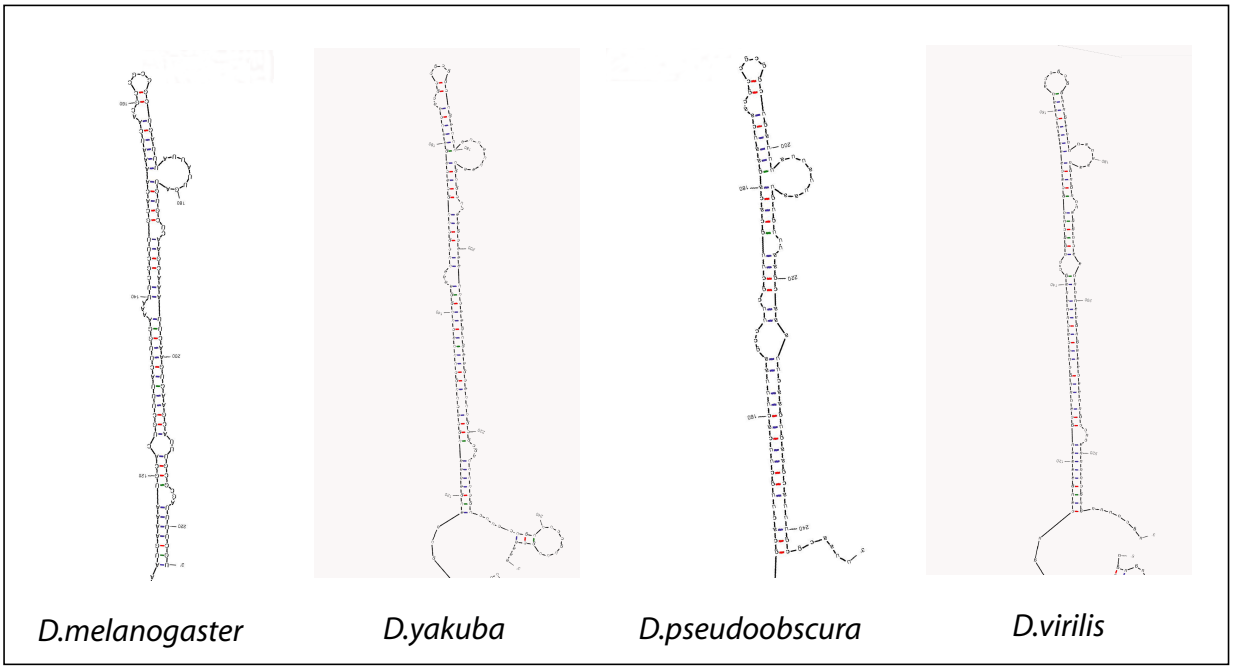


Figure 8: Similar secondary structure of dimerisation domains from orthologous *oskar* RNAs.
Comparison of RNA secondary structure models of *oskar* 3'UTR orthologs predicted with mfold (v2.3, 25°C). Only the conserved dimerisation domain (SLII) of *D.melanogaster, D.yakuba, D.pseudoobscura* and *D.virilis* is shown. For each ortholog, the palindromic sequence (-CCGCGG-) is predicted to form the terminal loop. The length of the stem-loop structure varies from 95 (*D.pseudoobscura*) to 114 (*D.yakuba*) nucleotides.

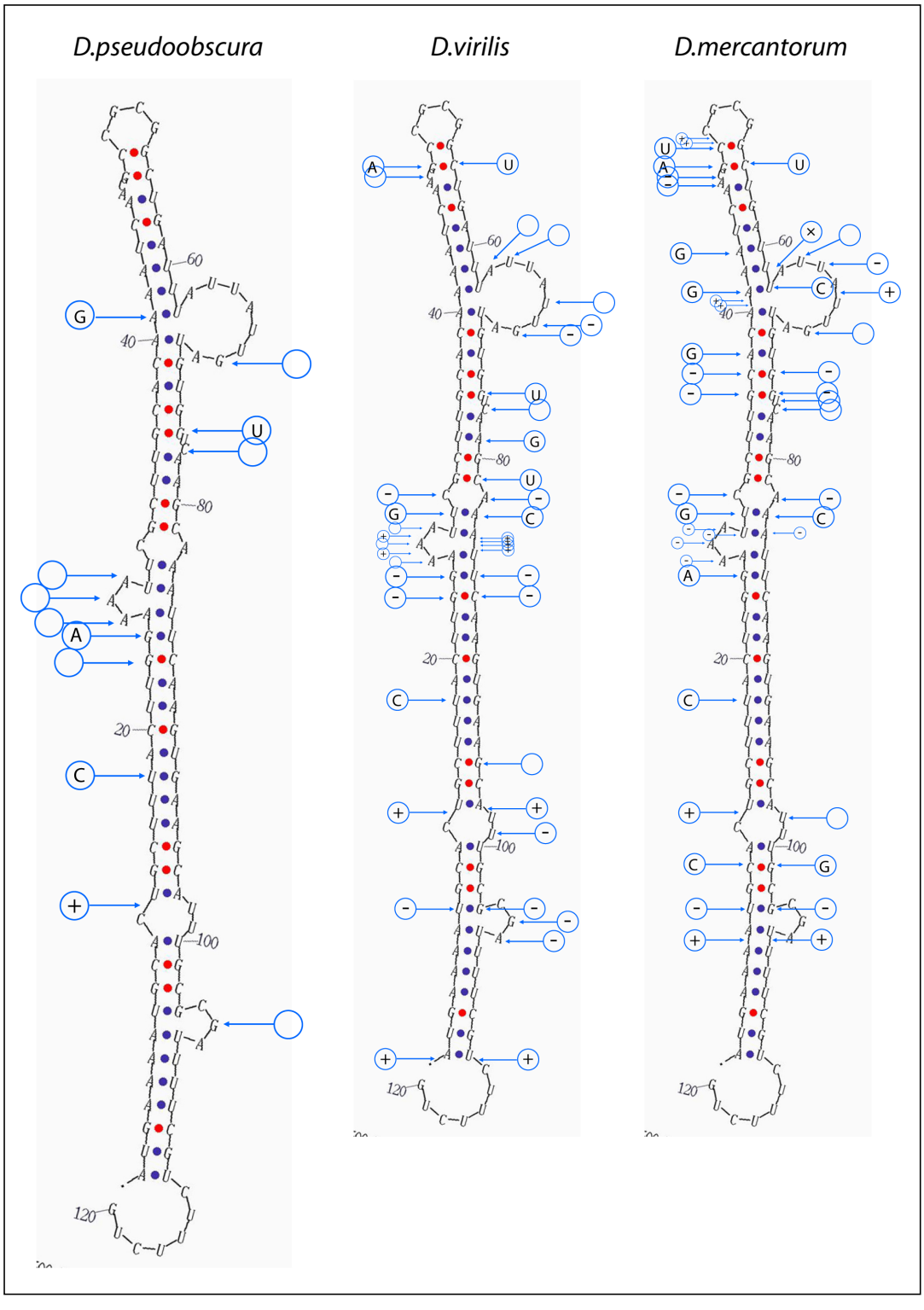


Figure 9: The *oskar* dimerisation domain shows little sequence variation between species.
Comparison of the position of nucleotide changes along the *oskar* dimerisation domain. The secondary structure of the *D.melanogaster* dimerisation domain is shown and all nucleotides that differ in other species are indicated. The nucleotide changes in *D.pseudoobscura* (A), *D.virilis* (B) and *D.mercantorum* (C) are indicated as follows: (-) marks the absence of a nucleotide and (+) indicates the presence of a nucleotide in the respective *oskar* orthologs. Nucleotide substitutions are either indicated as (when affecting the secondary structure), or as (G) (indicating a nucleotide change that does not affect the secondary structure, here a nucleotide change to guanine as an example is shown).

3.2. The dimerisation domain promotes *in vivo* interaction of *oskar* 3'UTRs

3.2.1. Constructing transgenes for *in vivo* analysis

To localise correctly at the posterior pole *oskar* mRNA requires both its intact 3'UTR as well as splicing at intron position 1 (Kim-Ha et al. 1993; Hachet and Ephrussi 2004). Indeed, Hachet and Ephrussi also showed that, challenging previous data, the *oskar* 3'UTR by itself is not sufficient for posterior localisation (Hachet and Ephrussi 2004). However in the same study they demonstrated that the *oskar* 3'UTR is able to accumulate at the posterior pole indirectly, if localisable *oskar* RNA is also present in the oocyte. The basis for this mechanism remained to be investigated. *In vitro* it was shown that *oskar* 3'UTR can dimerise via a stem-loop that is strongly conserved among *Drosophila* species (Christine Brunel, unpublished; this study, above). This led us to hypothesise that the hitch-hiking mechanism for posterior localisation of *oskar* 3'UTR, might be mediated by direct RNA-RNA interaction, via the dimerisation domain.

To test if the dimerisation domain has a role in hitch-hiking *in vivo*, I analysed posterior accumulation of intronless RNAs bearing either the wild-type *oskar* 3'UTR or, alternatively, *oskar* 3'UTRs with mutations in the dimerisation domain (see scheme in Figure 10). To allow hitch-hiking, the transgenic *oskar* 3'UTR RNAs were expressed in flies containing endogenous, wild-type *oskar* mRNA. In order to distinguish the transgenic *oskar* 3'UTR from the endogenous *oskar*, in *in situ* hybridisation experiments I fused the transgenic *oskar* 3'UTRs to the GFP ORF. By using a *gfp* antisense probe, the chimeric *gfp-oskar* RNA could unambiguously be detected in the egg chambers. The *gfp* antisense probe gave no signal in control oocytes of flies not expressing transgenic *gfp-oskar* RNAs. Furthermore, *gfp* RNA lacking the *oskar* 3'UTR neither accumulated in the oocyte nor hitch-hiked to the posterior pole (data not shown). Fusing the *gfp* ORF to the *oskar* RNA has no effect on the secondary structure of the *oskar* 3'UTR, as predicted by *mfold* (data not shown).

The mutations I analysed in the *oskar* 3'UTR dimerisation domain consisted of substitutions in the palindromic sequence that either replaced four nucleotides ("*gfp-oskar* 3'UTR-GNRA") or two nucleotides ("*gfp-oskar* 3'UTR-UU") in the loop of the dimerisation domain. Also analysed was a deletion removing 67 basepairs of the distal part of the stem (*gfp-oskar* 3'UTR-ΔSLIId; Figure 10).

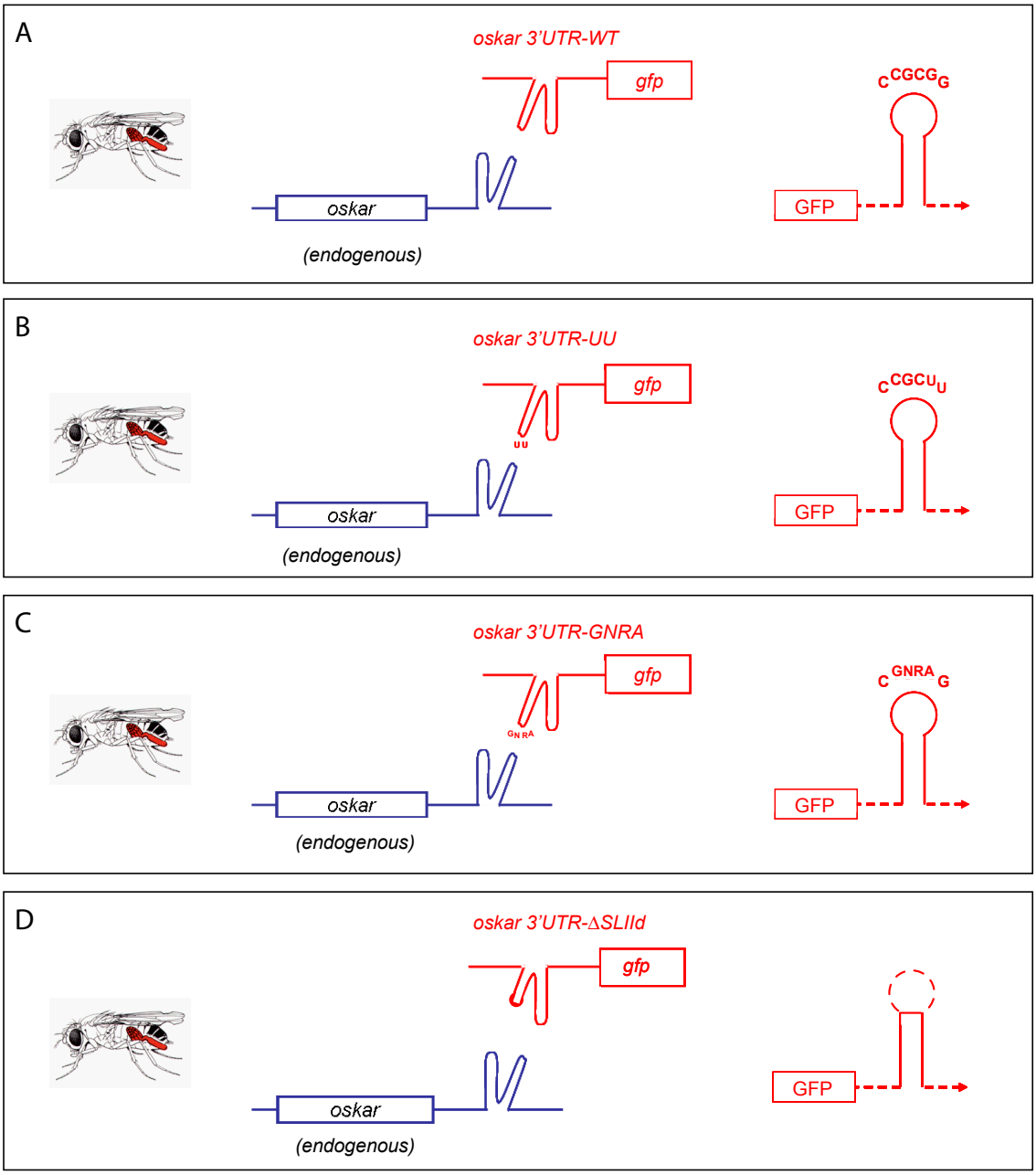


Figure 10: Schematic of experiments performed in section 3.2.
oskar 3'UTR transgenes (in red) were expressed in wild-type flies containing endogenous *oskar* mRNA (in blue). I then scored posterior hitch-hiking of the *oskar* 3'UTR RNA, along with endogenous *oskar*. The following transgenes were analysed: *oskar* 3'UTR-WT (a), *oskar* 3'UTR-UU (b), *oskar* 3'UTR-GNRA (c) and *oskar* 3'UTR-ΔSLIId (d).

In *in-situ* hybridisation experiments low amounts of RNA can remain undetected even at the site of localisation. Therefore, to reliably quantify and compare the localisation of transgenic *gfp-oskar* 3'UTRs at the posterior pole of oocytes, lines with comparable expression were needed. However, standard P-element mediated insertion of pUASp-constructs into the genome of recipient flies is random, and the expression of transgenes can be strongly influenced by position effects (Rubin and Spradling 1982). Therefore, the expression levels of several insertions of each transgenic construct were analysed by qRT-PCR using *gfp* specific primers. The lines shown in Figure 11A expressed highly similar levels of transgenic RNA and were used for further experimentation.

The localisation of the various *gfp-oskar* 3'UTR RNAs in early oogenesis (stages 2-8) was compared and, as a read-out for hitch-hiking, posterior accumulation was scored at stages 9 and 10, when endogenous *oskar* mRNA reaches the posterior pole and forms a tight crescent (Ephrussi et al. 1991; Kim-Ha et al. 1991).

3.2.2. Localisation of *oskar* 3'UTR RNA in young egg chambers is unaffected by mutations in the dimerisation domain

Endogenous *oskar* mRNA is localised in the developing oocyte from stage 2 of oogenesis onwards. At stages 2 to 7 it is found in the posterior half of the oocyte, while the nucleus (containing the condensed chromatin of the oocyte, the "karyosome") is localised in the centre. At stage 8 the nucleus has migrated to the anterior dorsal corner of the now triangular shaped oocyte (King, 1970). *oskar* mRNA is transiently localised at the anterior of the oocyte and most prominently in the dorsal corner, surrounding the nucleus. From stage 9 onwards, *oskar* mRNA is detected in a tight crescent at the most posterior pole of the oocyte (Ephrussi et al. 1991; Kim-Ha et al. 1991). All transgenic RNAs, with the exception of *oskar* 3'UTR-ΔSLIId RNA (whose localisation is discussed in section 3.2.5), localise like endogenous *oskar* mRNA at stages 2 to 8. The RNAs are enriched in the oocyte from stage 2 onwards and transiently accumulate at the anterior at stage 8, as revealed by whole mount *in situ* hybridisation (Figure 11B). Furthermore, no difference in localisation was observed between *oskar* 3'UTR-WT, -UU and -GNRA RNAs up to stage 8 (Figure 11B). This shows that nucleotide substitutions in the loop of the dimerisation domain do not prevent accumulation of the chimeric RNA in stage 2 to 8 oocytes.

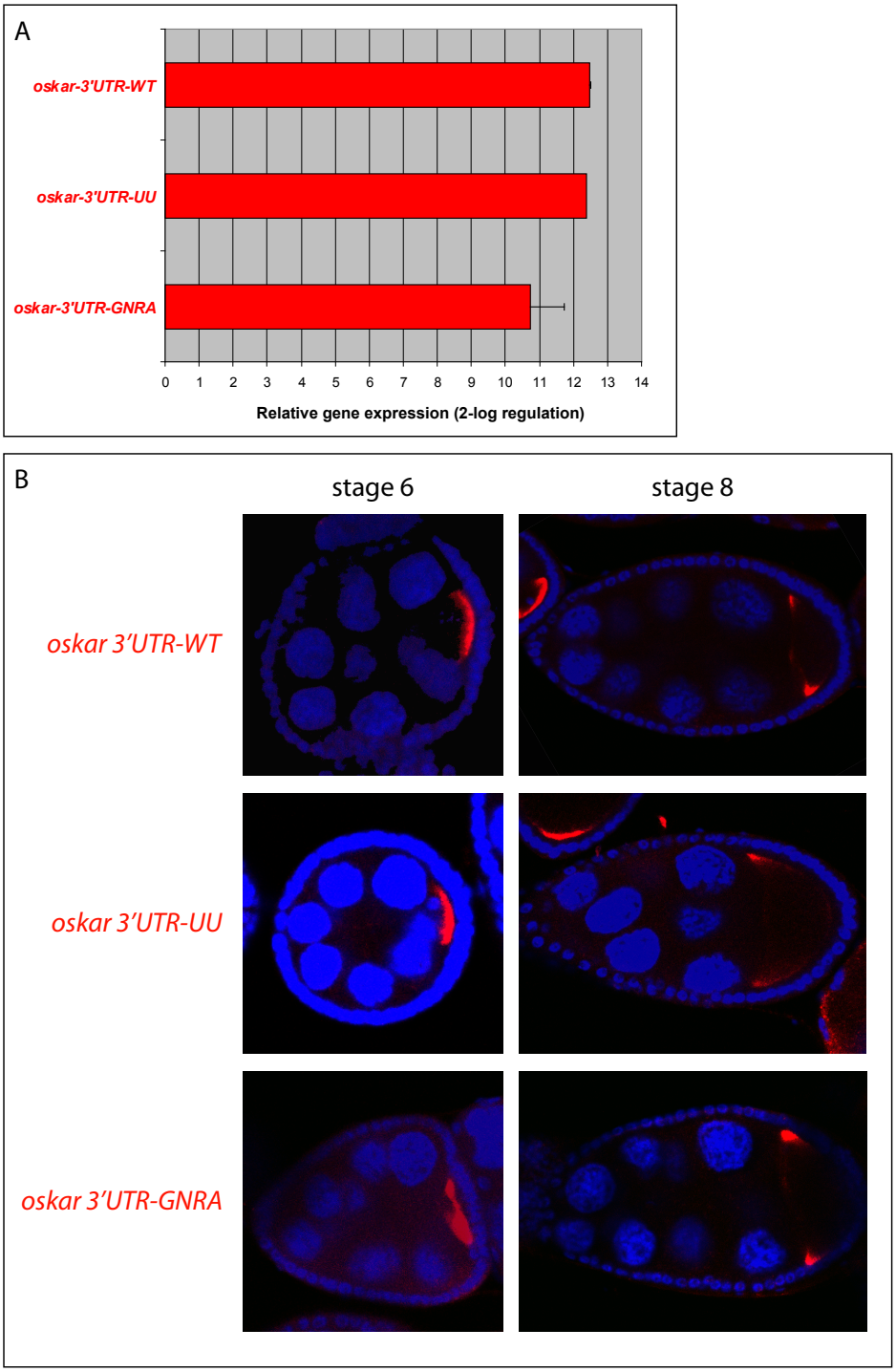


Figure 11: RNA expression levels and accumulation of the different *gfp-oskar* 3'UTR transgenes in young egg-chambers are comparable.

A. The relative expression of *gfp-oskar* 3'UTR transgenes was measured in quantitative RT-PCR experiments, using total RNA isolated from egg chambers. A primer set amplifying *gfp* RNA was used to detect the transgenic RNA, and the data were normalised to the expression levels of *rp49*. The graph shows RNA expression in 2-log scale relative to a negative control (*w1118* flies without the transgene). Shown are the relative expression of *gfp-oskar* 3'UTR-WT, (line 12), *gfp-oskar* 3'UTR-UU, (line 3) and *gfp-oskar* 3'UTR-GNRA (line 11). B. Accumulation of the *gfp-oskar* 3'UTR RNA in young oocytes analysed by *in situ* hybridisation. Shown are stages 6 and 8 egg-chambers expressing *gfp-oskar* 3'UTR-WT, -UU and –GNRA transgenes. The transgenic RNA was detected using an *gfp* antisense probe (in red) and the DNA (in blue) was stained with DAPI.

3.2.3. Mutations in the dimerisation domain impair posterior localisation of *oskar* 3'UTR *in vivo* at stage 9

The transgenes analysed were all equally expressed and their RNAs capable of localising in the oocyte. Next, their ability to hitch-hike to the posterior of the oocyte at stage 9 was analysed. *gfp-oskar* 3'UTR-WT RNA could be detected at the posterior pole in 87% of egg chambers, while in the case of *gfp-oskar* 3'UTR-UU and –GNRA RNAs, posterior accumulation was reduced to 39% and 31% respectively (Figure 12B).

To test if the poor ability of the mutated *oskar* 3'UTR RNAs to localise was due to a defect in posterior localisation of endogenous *oskar* mRNA, we simultaneously detected both *oskar* RNA and Staufen protein by in situ hybridisation coupled with immuno-detection. Staufen is a double-stranded RNA binding protein that specifically co-localises with *oskar* mRNA during oogenesis and is widely used as a marker for *oskar* mRNA localisation (St Johnston et al. 1991; Ramos et al. 2000). In the absence of *oskar* mRNA, Staufen protein is not localised at the posterior pole of the oocyte (St Johnston et al. 1991). Staufen associates with the *oskar* via the *oskar* 3'UTR, therefore in our experiment, Staufen presumably associates both with endogenous *oskar* mRNA and with the transgenic *oskar* 3'UTR RNA (Jenny, Hachet et al. 2006). By concurrently detecting *gfp* RNA and Staufen it was found that oocytes lacking posterior enrichment of *gfp-oskar* 3'UTR-GNRA and –UU nevertheless showed posterior Staufen (Figure 12A). We therefore deduced that in egg chambers lacking posterior *gfp-oskar* 3'UTR, endogenous *oskar* mRNA is correctly localised.

I confirmed that endogenous *oskar* mRNA was localised by *in situ* hybridisation using an antisense probe against the *oskar* coding region, thus specific for endogenous *oskar* RNA, which showed that neither of the transgenic RNAs affected the localisation of endogenous *oskar* (Figure 13). In addition, egg chambers were stained for Oskar protein. Oskar is exclusively expressed once *oskar* mRNA is localised at and restricted to the posterior pole from stage 9 onwards (Kim-Ha et al. 1995). Consequently the presence of Oskar at the posterior pole indicates that *oskar* mRNA is properly localised. Expression of the transgenic *oskar* 3'UTR RNAs had no effect on Oskar protein expression at stage 9, again showing that endogenous *oskar* mRNA regulation was unaffected at stages 9 and 10 of oogenesis (Figure 13).

These data shows that mutations in the dimerisation domain interfere with, but do not completely abolish localisation of a *gfp-oskar* 3'UTR reporter to the posterior pole at stage 9.

This therefore indicates to me that RNA-RNA interaction might play a role in the hitch-hiking process.

3.2.4. The localisation defects of the dimerisation mutants are partially rescued at stage 10

The posterior localisation of the transgenic *gfp-oskar* 3'UTR was also studied later in oogenesis, at stages 10A and 10B. Endogenous wild-type *oskar* mRNA remains at the posterior pole at these stages. The *gfp-oskar* 3'UTR-WT, as already observed at stage 9, co-localised with endogenous *oskar* mRNA at stages 10A and 10B, forming a posterior crescent in 97% of egg chambers (Figure 14). At these stages *oskar* 3'UTR-UU and –GNRA were predominantly detected at the posterior pole (UU: 87%, GNRA: 69% of egg chambers), showing only subtle localisation defects (Figure 14). This can be explained in two ways. First, hitch-hiking based localisation could be continuously employed at stages 9 and 10 and full interaction via the dimerisation loop would be necessary for "fast/efficient" hitch-hiking at stage 9. Alternatively, hitch-hiking to the posterior via the dimerisation domain might be a mechanism only employed at stage 9 and another 3'UTR-dependent mechanism might ensure localisation at stage 10.

3.2.5. *oskar* 3'UTR-ΔSLIId has severe defects enriching in young egg chambers

As mentioned in part 3.2.2, I also analysed *in vivo oskar* 3'UTR-ΔSLIId, a construct in which part of the stem of the dimerisation domain was deleted. The aim was to test whether this deletion, which includes nucleotides necessary for *in vitro* dimerisation, would have a stronger effect on hitch-hiking than nucleotide substitutions in the loop, possibly abolishing hitch-hiking altogether. This analysis was difficult as, although *oskar* 3'UTR-ΔSLIId was transcribed at a level equal to the control *oskar* 3'UTR-WT (Figure 15 C), the RNA was only detectable in 40% of young oocytes (Figure 15A, summary of statistical analysis B). This observation suggests a possible role of the same conserved stem-loop in oocyte entry (see part II of this thesis). Despite this early defect in many oocytes, in the remaining oocytes I analysed posterior crescent formation at stages 9 and 10. In 17% of all stage 9 and 60% of all stage 10 egg chambers, *gfp-oskar* 3'UTR-ΔSLIId RNA was detected at the posterior pole (Figure 15 B). Therefore, complete deletion of the loop does not appear to further decrease

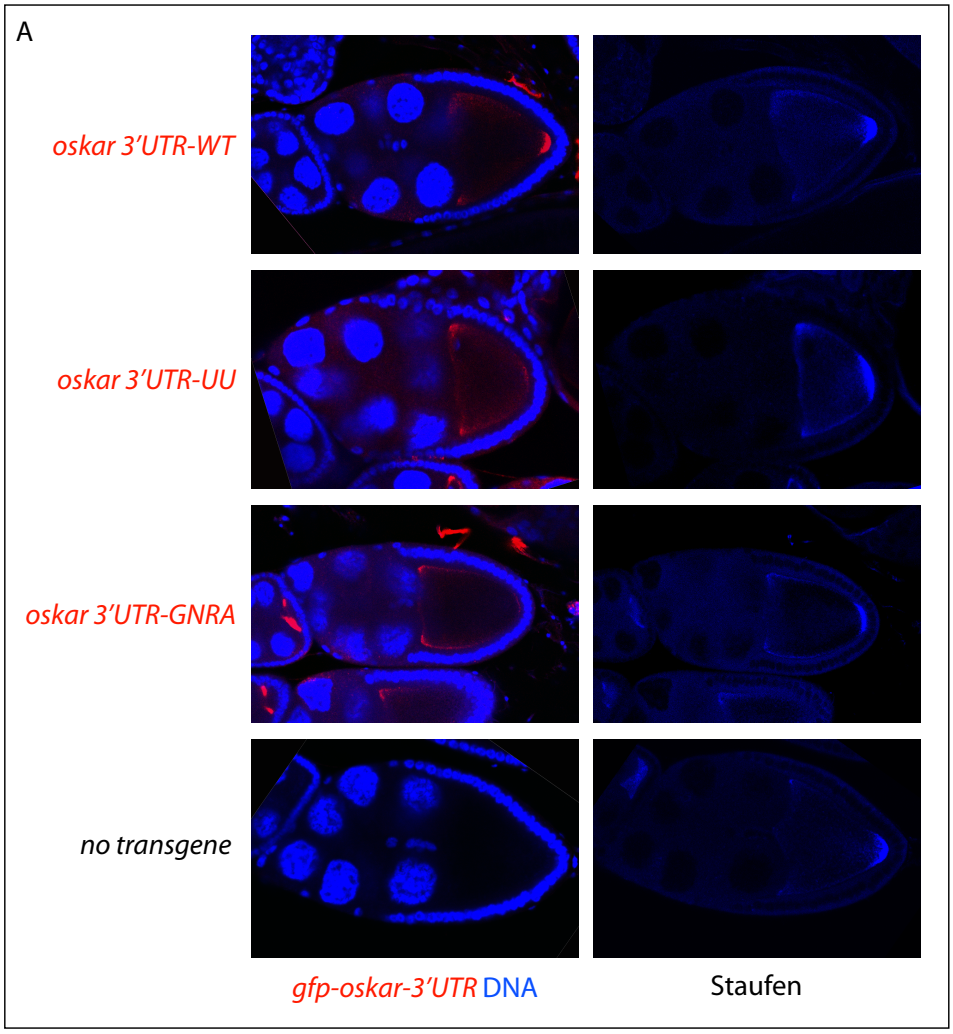


Figure 12: Mutations in the dimerisation domain reduce posterior accumulation of *oskar* 3'UTR RNA.

A. Stage 9 egg-chambers were stained for Staufen protein (in blue, right), DNA (in blue, left) and the transgenic RNA (in red, left) by FISH coupled with antibody staining. Shown are egg-chambers from flies without transgene (bottom) and flies expressing either *gfp-oskar* 3'UTR-WT, -UU or -GNRA. B. Summary of *in situ* hybridisation experiments detecting posterior *gfp* RNA. Shown are percentages of posterior accumulation at stage 9 for each transgene. The accumulation of *oskar* 3'UTR reporter RNA at the posterior pole was scored in at least two independent experiments, analysing at least 25 egg-chambers per experiment.

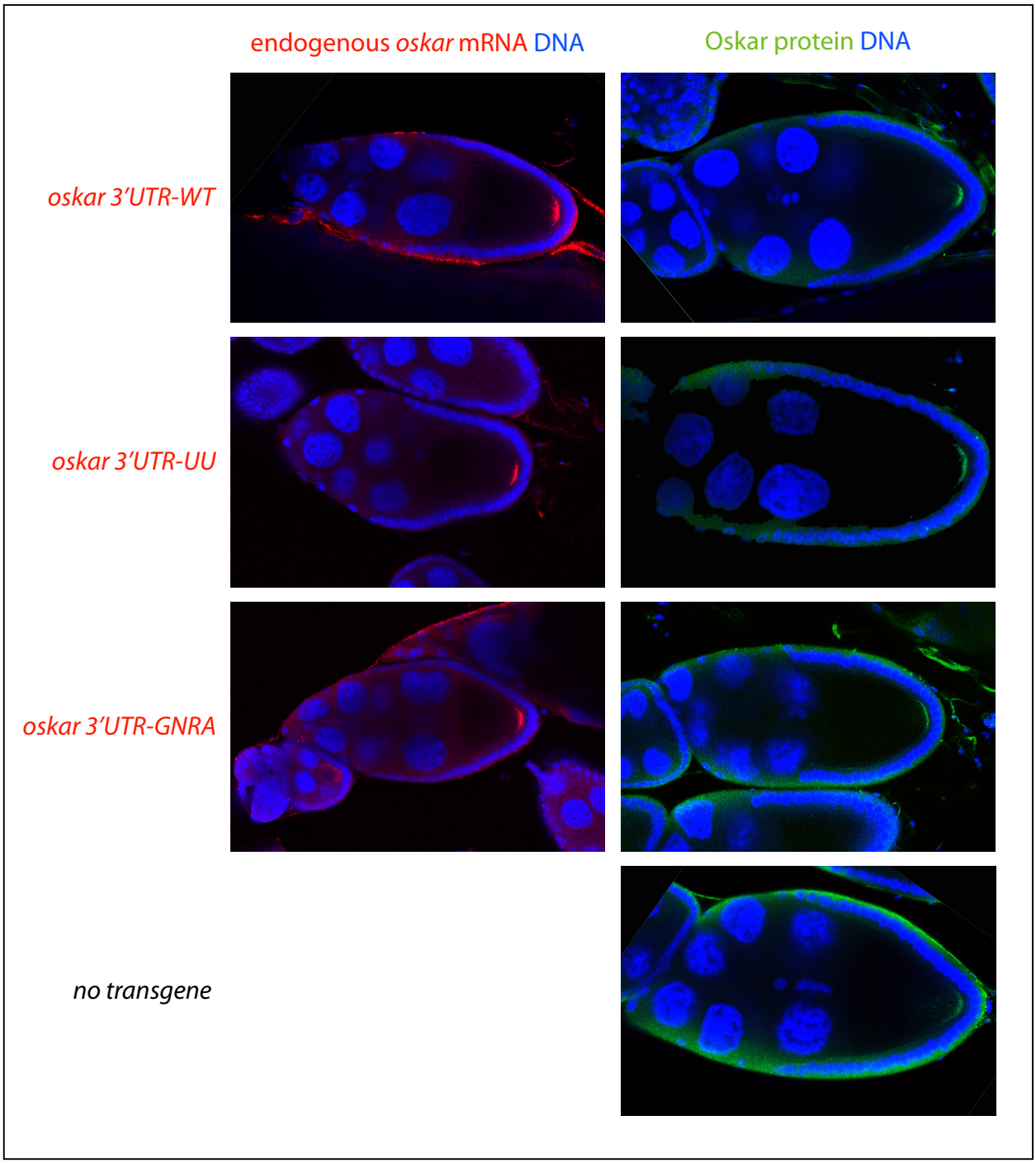


Figure 13: Localisation and expression of endogenous *oskar* RNA in mid-oogenesis is not affected by expression of transgenic RNA.
Egg chambers from flies expressing either *gfp-oskar* 3'UTR-WT, -UU or –GNRA were stained for endogenous *oskar* mRNA (in red) and Oskar protein (in green) and the DNA counterstained with DAPI (in blue). For comparison, flies not expressing the transgenic gfp-*oskar* 3'UTR were stained for Oskar protein. In each case, a stage 9 egg-chamber is shown.

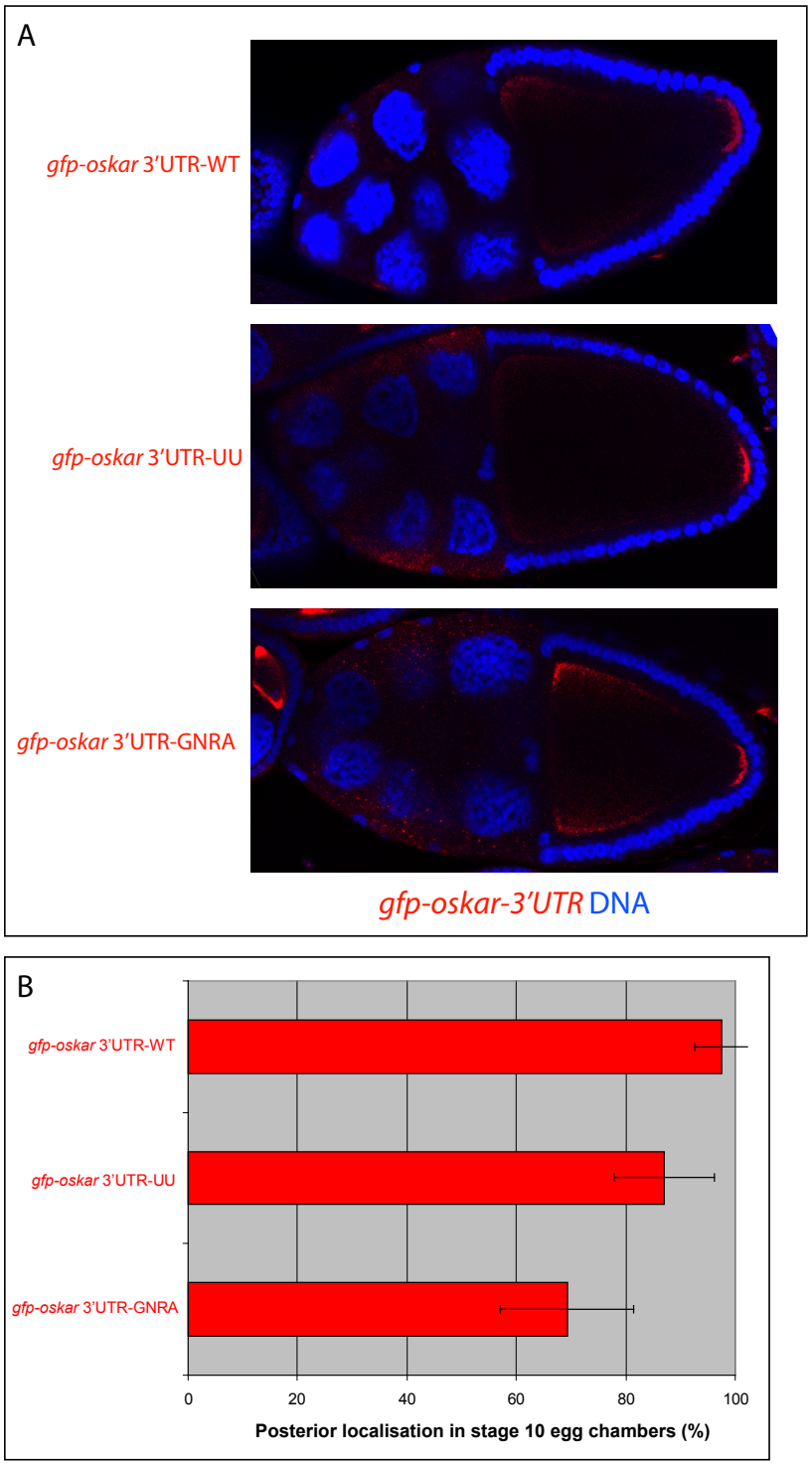


Figure 14: Accumulation of *oskar* 3'UTR RNA is mildly reduced in stage 10 egg-chambers.

A. Posterior accumulation of transgenic *oskar* 3'UTR RNA (in red) at stage 10 of oogenesis in egg-chambers expressing either *gfp-oskar* 3'UTR-WT, -UU or -GNRA; DNA (in blue). B. Summary of *in situ* hybridisation experiments detecting posterior *gfp* RNA. Shown are percentages of posterior accumulation at stage 10 for each transgene. The accumulation of *oskar* 3'UTR reporter RNA at the posterior pole was scored in at least two independent experiments, analysing at least 25 egg-chambers per experiment.

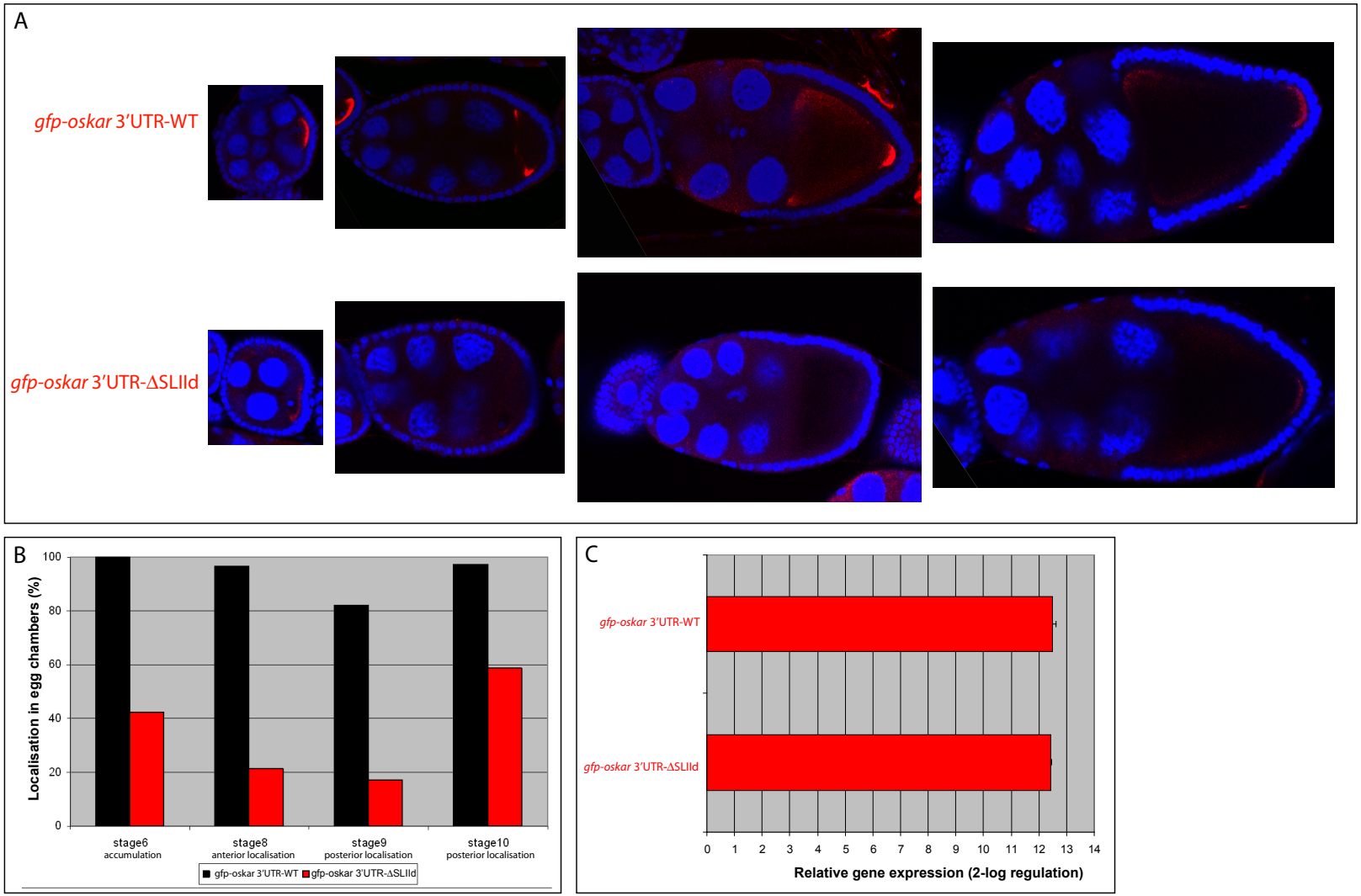


Figure 15: A 67-nucleotide deletion in the dimerisation domain reduces oocyte accumulation.
A. Comparing the accumulation of *gfp-oskar* 3'UTR-WT and -ΔSLIId RNAs at stages 6, 8, 9 and 10 of oogenesis by in situ hybridisation. Transgenic RNA (in red) was detected using a *gfp* antisense probe and the DNA (in blue) with DAPI. B. Summary of the observations from *in situ* hybridisation experiments. Shown are percentages of oocyte enrichment (stage 6 and 8) or posterior localisation (stage 9 and 10). C. The relative gene expression levels of the transgenes (gfp-oskar 3'UTR-WT and ΔSLIId) were measured by quantitative RT-PCR, using a primer set that specifically amplifies *gfp* RNA. Transgene expression was normalised to *rp49* expression, and is shown in 2-log scale relative to a negative control.

posterior localisation at stages 9 and 10, beyond the effect of nucleotide substitutions in the loop. As I could still observe some posterior hitch-hiking of *oskar* 3'UTR-ΔSLIId RNA, I conclude that hitch-hiking does not solely depend on the dimerisation domain.

3.2.6. Interaction via the SLII-loop is not sufficient for hitch-hiking

In vitro the dimerisation domain is sufficient for dimerisation and *in vivo* it is involved in hitch-hiking. To investigate whether a 272 basepair region including the dimerisation domain ("SLI-II") is sufficient for hitch-hiking, I analysed transgenic flies expressing this region fused to *gfp* alone. *gfp*-SLI-II RNA can accumulate during early oogenesis in the oocyte (stages 2 to 7), but fails to enrich at the posterior pole in mid oogenesis (stages 9-10) (Figure 16 A). The relative amounts of mRNA expressed from several *gfp*-SLI-II transgene insertions were quantified and were found to be similar to that of the *gfp-oskar* 3'UTR-WT (Figure 16 B). These qRT-PCR data, together with the observation that SLI-II is strongly enriched in early oocytes, show that SLI-II mRNA is stably expressed in the germline. The lack of posterior enrichment therefore can not be attributed to poor expression of the SLI-II transgene. The conclusion therefore must be that SLI-II is not sufficient for hitch-hiking with endogenous *oskar* mRNA to the posterior pole. Both these data and the analysis of *oskar* 3'UTR-ΔSLIId, suggest that in addition to the dimerisation domain another, yet to be characterised, region of the *oskar* 3'UTR also contributes to hitch-hiking *in vivo*.

3.2.7. Hitch-hiking is promoted by direct RNA-RNA interaction of *oskar* molecules

In vitro dimerisation of *oskar* 3'UTR RNA depends on nucleotide interaction via the palindromic loop of the SLI-II region. Substitutions in this loop strongly reduce the RNA interaction, but combining *oskar* 3'UTR RNAs bearing complementary mutations in trans fully restores dimerisation. *In vivo*, hitch-hiking is impaired by substitutions in the loop of the dimerisation domain, but it is unclear whether this reflects a direct or indirect RNA interaction. An indirect interaction would be mediated by a factor (RNA or protein) binding simultaneously to two *oskar* mRNA molecules. It is known that *oskar* RNA is found in heavy particles containing RNA and protein. At least two proteins, Bruno (Chekulaeva et al. 2006) and PTB (F.Besse and S.Lopez de Quinto, unpublished) have been identified, each of which could mediate oligomeriserisation of *oskar* mRNA *in vivo*. On the other hand, a direct

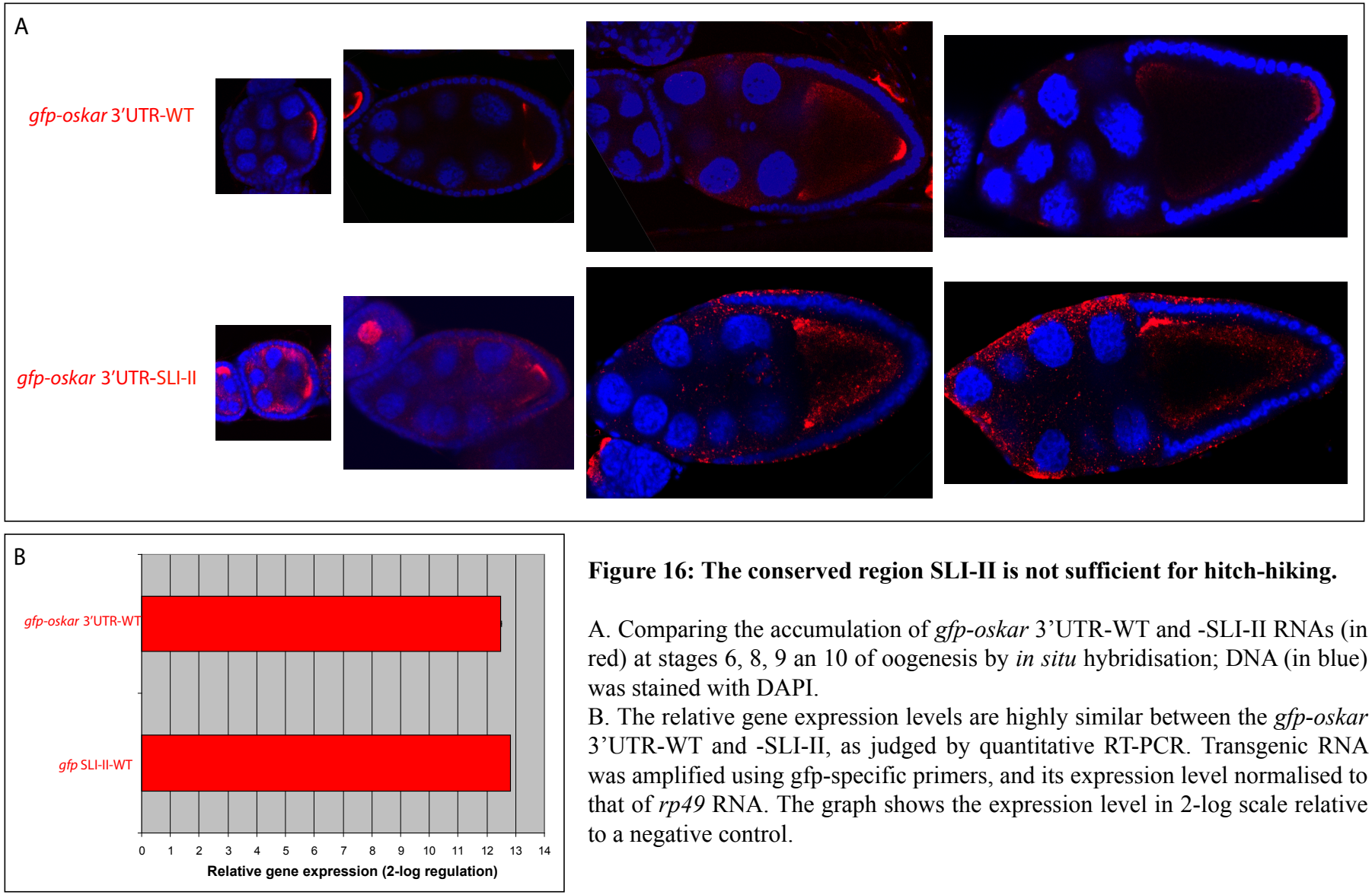


Figure 16: The conserved region SLI-II is not sufficient for hitch-hiking.

A. Comparing the accumulation of *gfp-oskar* 3'UTR-WT and -SLI-II RNAs (in red) at stages 6, 8, 9 an 10 of oogenesis by *in situ* hybridisation; DNA (in blue) was stained with DAPI.
B. The relative gene expression levels are highly similar between the *gfp-oskar* 3'UTR-WT and -SLI-II, as judged by quantitative RT-PCR. Transgenic RNA was amplified using gfp-specific primers, and its expression level normalised to that of *rp49* RNA. The graph shows the expression level in 2-log scale relative to a negative control.

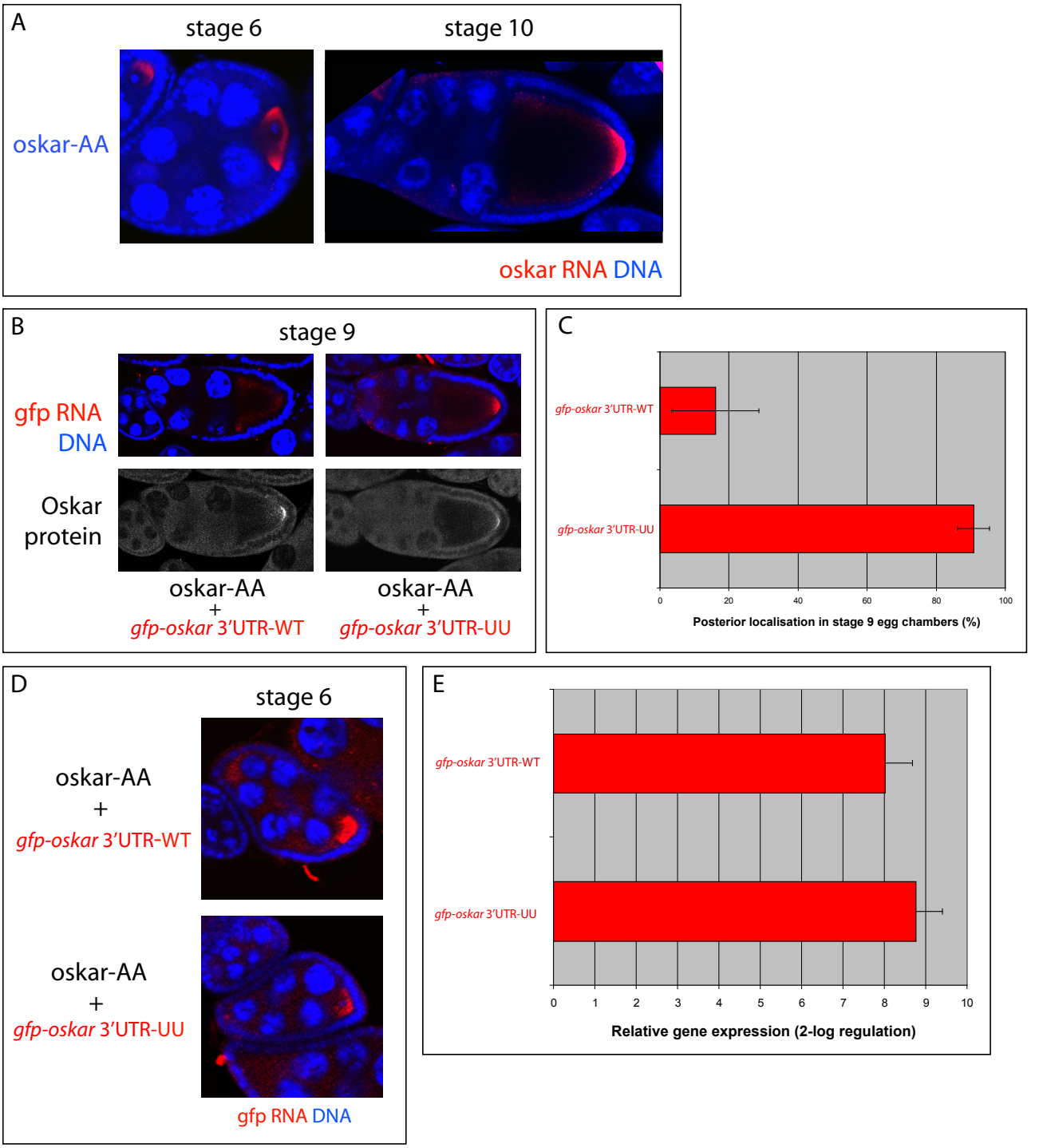


Figure 17: ***oskar*** **molecules interact directly via their dimerisation domains.**
A. Transgenic *oskar-AA* RNA is enriched in stage 6 oocytes and localises at the posterior pole (stage10). The transgenic *oskar-AA* (in red) was expressed in *oskar* RNA null flies and detected by i*n situ* hybridisation, using an antisense probe specific for the *oskar* coding sequence. B/C/D/E. Egg-chambers of *oskar* RNA null flies co-expressing *oskar-AA* and *gfp-oskar* 3'UTR-WT or –UU. B. At stage 9 of oogenesis, *oskar-AA* mRNA was indirectly detected by staining for Oskar protein (in white, right), while the *gfp-oskar* 3'UTR RNAs (in red, left) were simultaneously detected using an antisense gfp probe. C. Summary of posterior accumulation of *gfp-oskar* 3'UTR-WT and -UU at stage 9 of oogenesis. D. *gfp-oskar* 3'UTR-WT and -UU RNA (in red) accumulation in young stages, detected by *in situ* hybridisation. E. The relative gene expression levels of both transgenic *gfp-oska*r 3'UTRs measured by quantitative RT-PCR. The transgenic RNAs were amplified using primers directed against the *gfp* portion of the RNA and normalised to expression levels of *rp49*. The graph shows the expression level in 2-log scale relative to a negative control.

interaction would allow two *oskar* molecules to form an RNA-RNA dimer or, if multiple regions interact, an oligomere. I hypothesised that if *in vivo* hitch hiking is based on direct RNA-RNA interaction, then compensatory mutations in the dimerisation domain should restore hitch-hiking, similar to the *in vitro* observations.

To test this hypothesis I generated an *oskar* transgene bearing a 2-nucleotide AA substitution in the loop if the dimerisation domain ("*oskar*-AA"). A prerequisite for studying hitch-hiking along with this *oskar*-AA is that the RNA itself must localise to the posterior of the oocyte. Endogenous *oskar* mRNA localisation to the posterior pole depends on the 3'UTR and splicing of the pre-mRNA at intron position 1. Therefore the AA-substitution was analysed in the context of genomic *oskar*, to allow splicing of the RNA and posterior localisation. As shown by *in situ* hybridisation, genomic *oskar-AA* localised at the posterior like wild-type *oskar* (Figure 17 A). For a more detailed description of the genomic *oskar-AA* phenotype see section 3.3.

Genomic *oskar*-AA is complementary to the substitution of the *gfp-oskar* 3'UTR-UU, and both RNAs should be capable of interacting via the dimerisation domain. I next co-expressed genomic *oskar*-AA with either the *gfp-oskar* 3'UTR-UU or alternatively with *gfp-oskar* 3'UTR-WT. If indeed direct RNA interaction is involved in hitch-hiking, the compensatory mutated *gfp-oskar* 3'UTR-UU should hitch-hike to the posterior pole with *oskar*-AA, while *gfp-oskar* 3'UTR-WT should not. By *in situ* hybridisation I tested for accumulation of the *gfp-oskar* 3'UTR RNAs at the posterior pole of the oocyte at stage 9. To test for hitch-hiking of *gfp-oskar* 3'UTR RNAs with *oskar*-AA, the transgenes were co-expressed in flies lacking endogenous *oskar* mRNA (osk^{A87}/Df(3R)p^{XT103}, described in (Jenny, Hachet et al. 2006).

The capacity of the *gfp-oskar* 3'UTR-WT and -UU to hitch-hike with genomic *oskar*-AA to the posterior at stage 9 was then probed. Using *in situ* hybridisation and an antisense *gfp* probe, *gfp-oskar* 3'UTR-UU was localised to the posterior pole in 91% of oocytes, while posterior *gfp-oskar* 3'UTR-WT only in 16% (Figure 17 C). Oskar protein is only translated at the posterior pole of stage 9 oocytes if *oskar* mRNA is correctly localised there. Therefore as a read-out for localised *oskar*-AA, only stage 9 egg chambers that showed posterior staining for Oskar protein were scored (Figure 17 B).

The difference in posterior accumulation between *gfp-oskar* 3'UTR-WT and –UU RNAs was not caused by a general expression problem of the 3'UTR-WT transgene: in young egg chambers (stage 2 to 7) both *gfp-oskar*-WT and -UU were similarly enriched in the oocyte

and the expression levels of the two RNAs were comparable in qRT-PCR experiments from ovarian RNA (Figure 17 D,E).

In summary, *oskar* 3'UTR-UU can hitch-hike to the posterior pole with *oskar*-AA, while hitch-hiking of *oskar* 3'UTR-WT is strongly reduced. I therefore conclude that *oskar* RNA molecules directly base-pair via their respective dimerisation domains in hitch-hiking *in vivo*.

3.3. The dimerisation domain is involved in translational repression of *oskar*

The hitch-hiking mechanism, based on direct RNA-RNA interaction, is necessary to fully localise RNAs bearing the *oskar* 3'UTR at the posterior pole at stage 9. Furthermore, hitch-hiking acts through nucleotides in the loop of the dimerisation domain. However, the biological significance of this RNA-RNA interaction for endogenous *oskar* mRNA remained unclear. I therefore analysed, whether mutating the dimerisation domain had an effect on the regulation of full-length *oskar* RNA. To this end, I expressed genomic *oskar*-AA (described in section 3.2.7), which cannot engage in loop-mediated dimerisation, and control *oskar*-WT in flies lacking endogenous *oskar* mRNA. Several aspects of *oskar* mRNA localisation and translational control were compared in *oskar*-AA and *oskar*-WT expressing flies (Figure 18).

3.3.1. *oskar* mRNA localisation is unaffected by a mutation in the dimerisation domain

In general, mis-regulation of *oskar* mRNA leads to a change in Oskar protein levels, which can either affect or even terminate embryogenesis (Ephrussi and Lehmann 1992; Smith et al. 1992). Even rather slight changes in *oskar* mRNA levels can already cause strong defects in development. It was therefore first ascertained, that *oskar*-AA and the control *oskar*-WT transgenes were expressed at similar levels. The RNA expression from both constructs was analysed by qRT-PCR of total RNA from ovaries and transgenic lines expressing the RNAs at similar levels at 25°C were selected for further study (Figure 19A). However, both mRNAs were 1.7 to 1.8 fold up-regulated relative to the level of *oskar* RNA expression in wild-type flies (w^{1118}) (Figure 19A, shown is the 2-log regulation normalised to *oskar* levels in w^{1118} flies).

Both *oskar*-AA and *oskar*-WT accumulated in the ovaries of young egg chambers at stage 6 and stage 8 and appeared to enrich similarly, as detected by in situ hybridisation using an *oskar* antisense probe (Figure 19B). At stage 9 of oogenesis, both transgenic RNAs were able to localise to the posterior pole, yet both showed reduced posterior accumulation compared to endogenous *oskar* mRNA (Figure 19C). However, the difference between *oskar*-WT and *oskar*-AA accumulation at the posterior pole was not significant (Figure 19D) and the

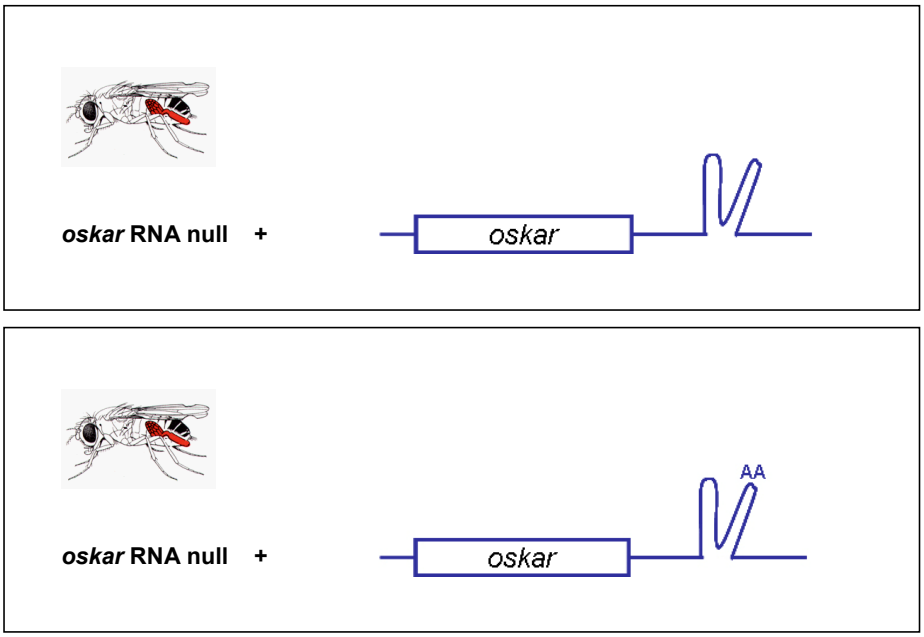


Figure 18: Schematic of experiments performed in section 3.3.
oskar-WT or *oskar-A*A transgenes (blue) were expressed i*n oskar* RNA null flies. I then analysed whether *oskar*-WT and *oskar*-AA were differently regulated. To this end I evaluated *oska*r mRNA localisation during oogenesis and protein expression in oogenesis and embryogenesis.

variance between several *oskar*-AA lines with similar expression levels was greater than between *oskar*-WT and *oskar*-AA (data not shown).

In summary, *oskar* mRNA localisation appears unaffected when mutating the loop in the dimerisation domain.

3.3.2. A mutation in the dimerisation domain causes *oskar* mis-expression

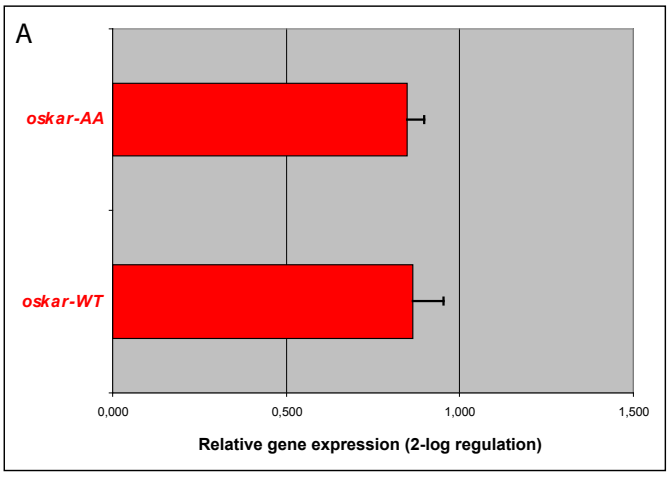
As *oskar-AA* is both enriched in and localised at the posterior pole of the oocyte, I next tested whether Oskar protein is expressed normally. A rapid assay to test for normal Oskar protein levels is to analyse the hatching rates of embryos. If embryos have abnormal levels of Oskar protein, they do not hatch at all or have a reduced hatching rate (Lehmann and Nusslein-Volhard 1986; Markussen et al. 1995). Females expressing *oskar-AA* laid wild-type looking eggs, however only 2% of these developed normally and hatched. In comparison, 59% of eggs expressing *oskar*-WT hatched (Figure 20A). This suggests that expressing *oskar*-AA strongly perturbs embryogenesis. As low hatching rates can have different causes, to gain more insight into the basis for the reduced hatching rate, I analysed the denticle pattern of non-hatching embryos. Insect denticles are cuticular protrusions produced by ventral cells in the embryo epithelium and are visible once the egg-shell is removed. Each segment has a band of denticles that is important for larval locomotion. Variations in Oskar protein levels usually result in stereotypical changes in embryonic patterning that are manifest as aberrant patterns in the denticle belts formed in late embryogenesis. If embryos have reduced Oskar protein levels, the denticle belts are fused or, in more severe cases, embryos lack all abdominal denticles (Lehmann and Nusslein-Volhard 1986). In contrast a mild ectopic expression of Oskar leads to defects in head structures, while in embryos with high levels of ectopic Oskar protein, the head is completely replaced by a duplication of the posterior structures called “bicaudal” (Ephrussi and Lehmann 1992; Smith et al. 1992).

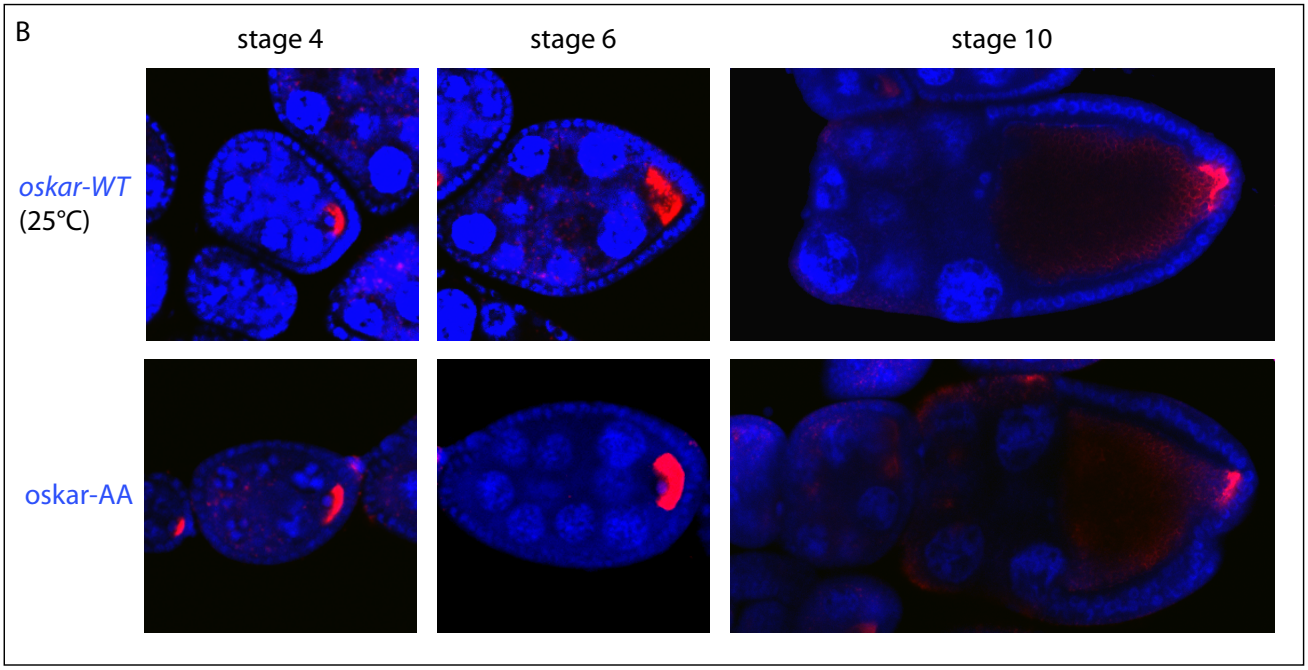
Of the non-hatching embryos that expressed the transgenic *oskar*-WT RNA, 24% displayed anterior patterning defects indicative of ectopic Oskar protein expression (Figure 20B). This was probably caused by the elevated *oskar* mRNA levels (see Figure 19A), previously shown to result in Oskar over-expression (Ephrussi and Lehmann 1992; Smith et al. 1992). In contrast, nearly all un-hatched embryos expressing *oskar*-AA showed strong anterior patterning defects (99%, Figure 20B), suggesting that translational repression of *oskar*-AA was severely impaired.

Both a very strong over-expression of Oskar at the posterior pole and premature expression of unlocalised *oskar* mRNA at the anterior pole can lead to ectopic Oskar activity and anterior patterning defects. To investigate when during oogenesis *oskar-AA* translation is mis-regulated, egg chambers expressing *oskar*-WT or *oskar*-AA were stained for Oskar protein. Although *oskar*-WT and *oskar*-AA mRNAs localised equally well at the posterior pole (~90% of egg chambers, see Figure 19C,D), the Oskar protein distribution resulting from expression of these RNAs was strikingly different. In *oskar-WT* expressing flies Oskar protein was not detected in stages 2 to 8 of oogenesis (Figure 21 B) and was restricted to the posterior pole of the oocyte from stage 9 onwards (Figure 21 C, D). In contrast, Oskar protein expressed from *oskar-AA* mRNA was already detected at stage 8 (Figure 21 B). From stage 9 onwards Oskar expressed from *oskar*-AA transgenes formed the normal posterior crescent and was additionally detected ectopically around the entire cortex of the oocyte (Figure 21 C,D). In approximately 30% of egg chambers *oskar*-WT and -AA mRNAs were present both at the posterior pole and in an additional ectopic patch in the centre of the oocyte. Simultaneous detection of *oskar* mRNA and protein revealed that unlocalised *oskar-WT* mRNA is not translated (Figure 21 C) but ectopic *oskar-AA* mRNA always co-localises with Oskar protein (Figure 21 C).

oskar mRNA encodes two isoforms, Short Oskar (55 kDa) and Long Oskar (71 kDa) with distinct functions (Markussen et al. 1995; Vanzo and Ephrussi 2002). To test whether both Oskar isoforms were up-regulated, a western blot using ovarian extracts of *oskar*-WT and *oskar*-AA flies was probed with anti-Oskar antibodies. As shown in Figure 21A, both isoforms of Oskar were clearly over-expressed in *oskar-AA*-expressing flies compared to *oskar*-WT flies.

Altogether, these results show that *oskar*-AA is prematurely and ectopically translated, causing anterior patterning defects and embryo lethality. This suggests that nucleotides in the loop of the dimerisation domain are critical for *oskar* repression.





C

	Posterior crescent	Posterior and ectopic localisation	Not localised
oskar-WT (25°C)	63%	30%	8%
oskar-AA	52%	39%	9%

Figure 19: RNA expression levels and accumulation in egg chambers are comparable between *oskar-WT* and *oskar-AA* transgenes.
A,B,C. *oskar-WT* or *oskar-AA* transgenic RNAs were expressed in *oskar* RNA null flies. A. The relative gene expression levels measured in quantitative RT-PCR experiments are highly similar between the *oskar-WT* and *oskar-AA*. Transgenic RNA was amplified using primers directed against oskar mRNA (exons 3-4) and normalised to expression levels of *rp49*. The graph shows the expression level in 2-log scale relative to the level of endogenous *oskar* in *w1118* flies. B, C. Oocyte accumulation of *oskar* is not affected by the AA-substitution in the dimerisation domain. In B, egg-chambers of stage 4 and 6 and in C, egg-chambers of stage 9 are shown, stained with an *oskar* antisense probe (in red) and DAPI (in blue). D. Summary of mRNA *oskar-WT* and *oskar-AA* localisation in oocytes at stage 9.

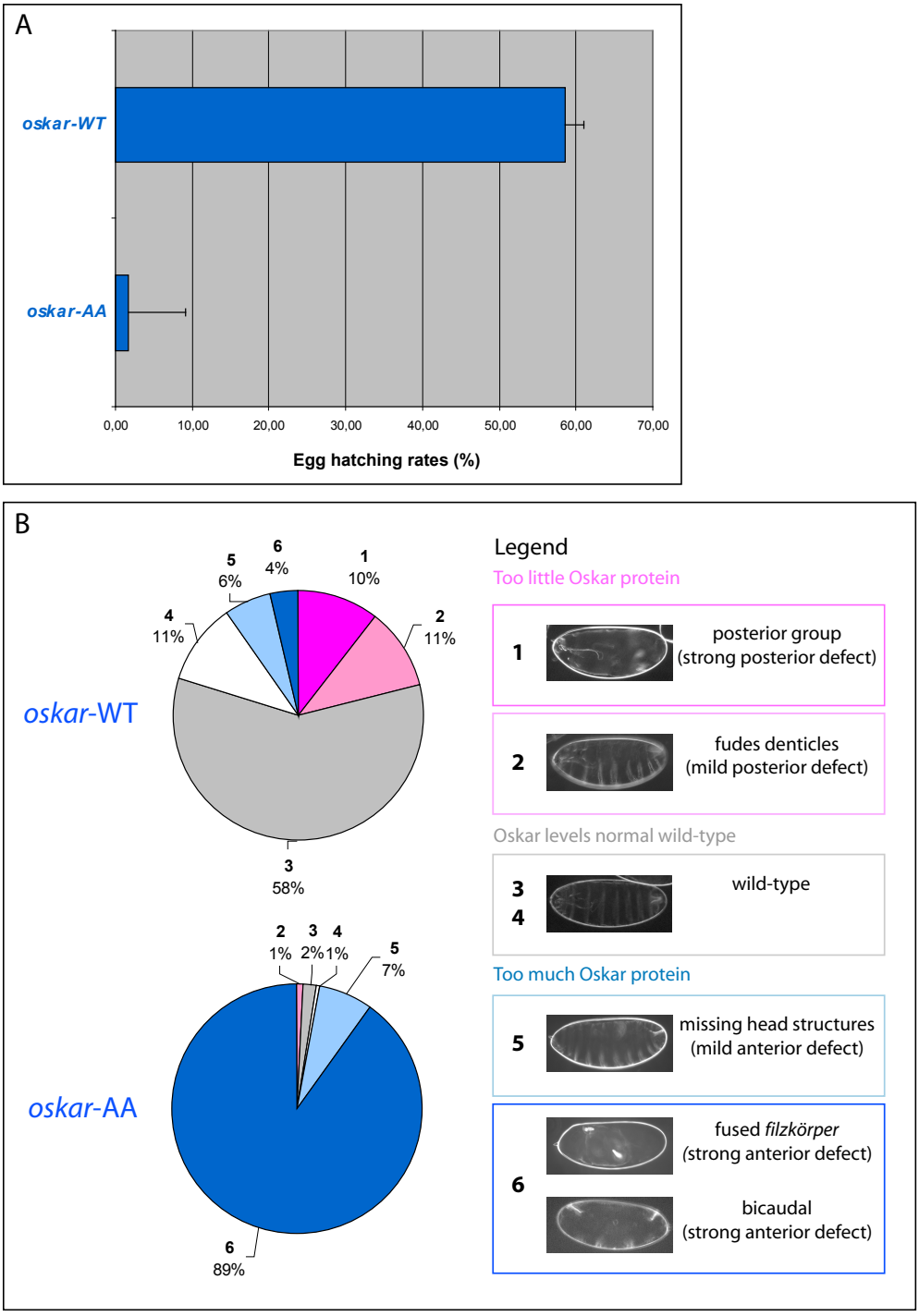


Figure 20: Oskar protein expression is mis-regulated in embryos expressing *oskar-AA*.
A. The hatching rates of eggs laid by females expressing *oskar*-WT or *oskar*-AA were compared, and *oskar*-AA expression found to strongly reduce embryonic survival. B. Analysis of the cuticle pattern of un-hatched embryos. The following embryonic phenotypes were scored: mild and strong "posterior group" phenotypes (pink), mild anterior head defects (pale blue), and "bicaudal" embryos (dark blue). The wild-type embryos were classified as hatched (white) or un-hatched (grey).

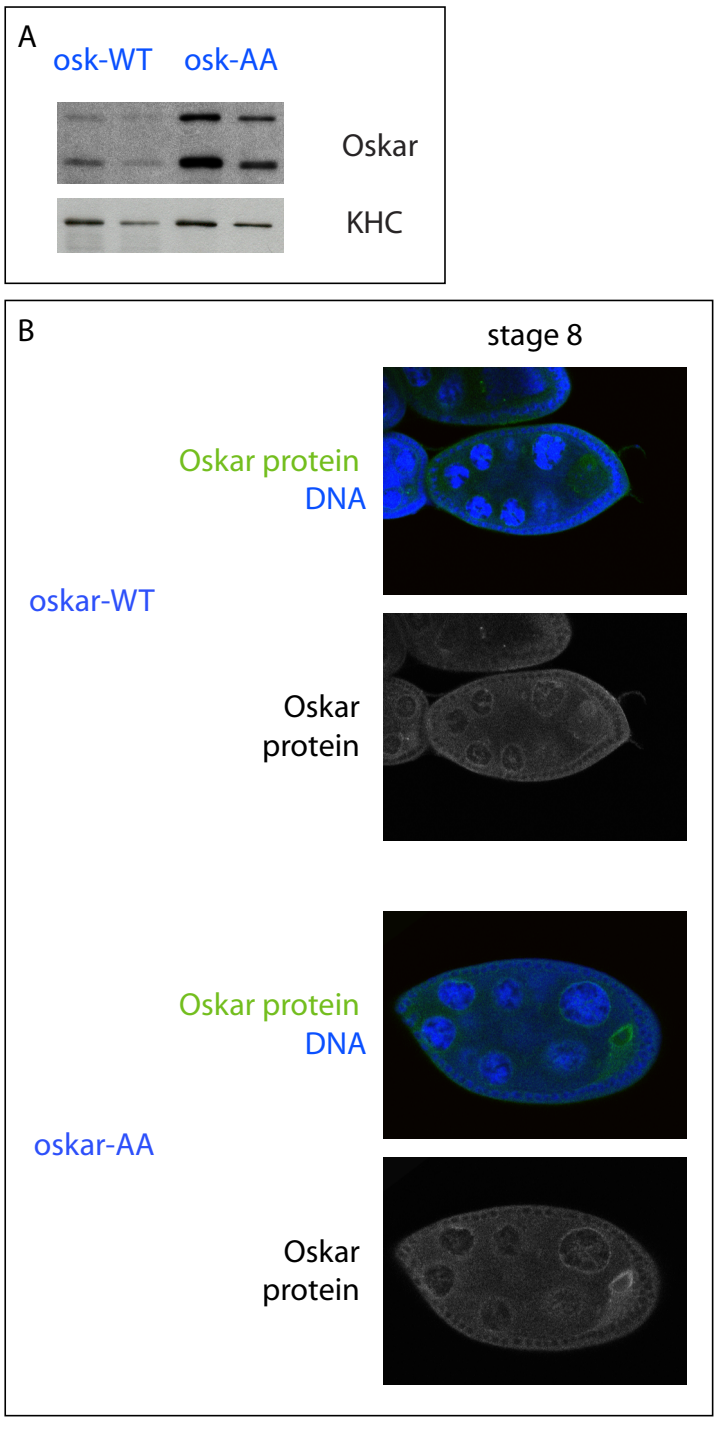
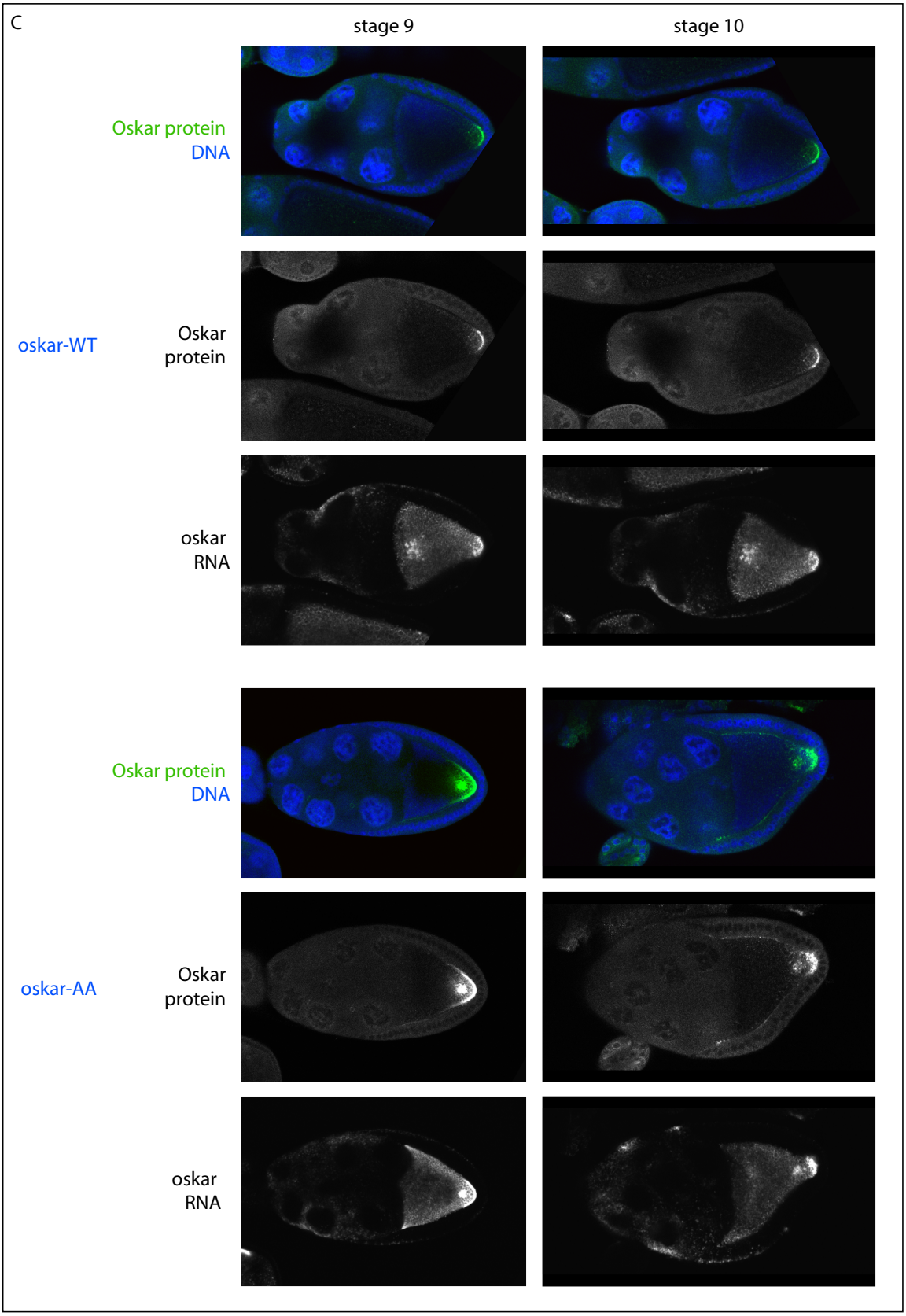


Figure 21: Oskar protein levels are up-regulated in *oskar*-AA expressing ovaries.
A. Different amounts of ovarian (egg chamber) protein extract were probed with anti-Oskar antibody in western blot experiments. Compared to the *oskar*-WT control, both isoforms of Oskar protein are up-regulated when expressed from *oskar*-AA mRNA in ovaries. Kinesin served as a loading control.

B, C, D. Staining of egg chambers expressing either *oskar*-AA or the control *oskar*-WT transgene in *oskar* RNA null flies. Oskar protein (in green/white) and *oskar* RNA (white) were detected by FISH coupled with antibody staining; DNA (blue) was stained with DAPI. B. When expressed from the *oskar*-AA transgene, Oskar protein accumulates prematurely in stage 8 oocytes, while no Oskar protein is detected in egg-chambers from *oskar*-WT-expressing flies. C. At stages 9 and 10, un-localised *oskar*-WT mRNA is repressed, while from ectopic *oskar*-AA Oskar is expressed. D. In egg-chambers from *oskar*-AA flies, Oskar protein is detected around the whole oocyte cortex, while the protein is exclusively at the posterior pole in egg chambers expressing oskar-WT.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stage 9
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Oskar protein
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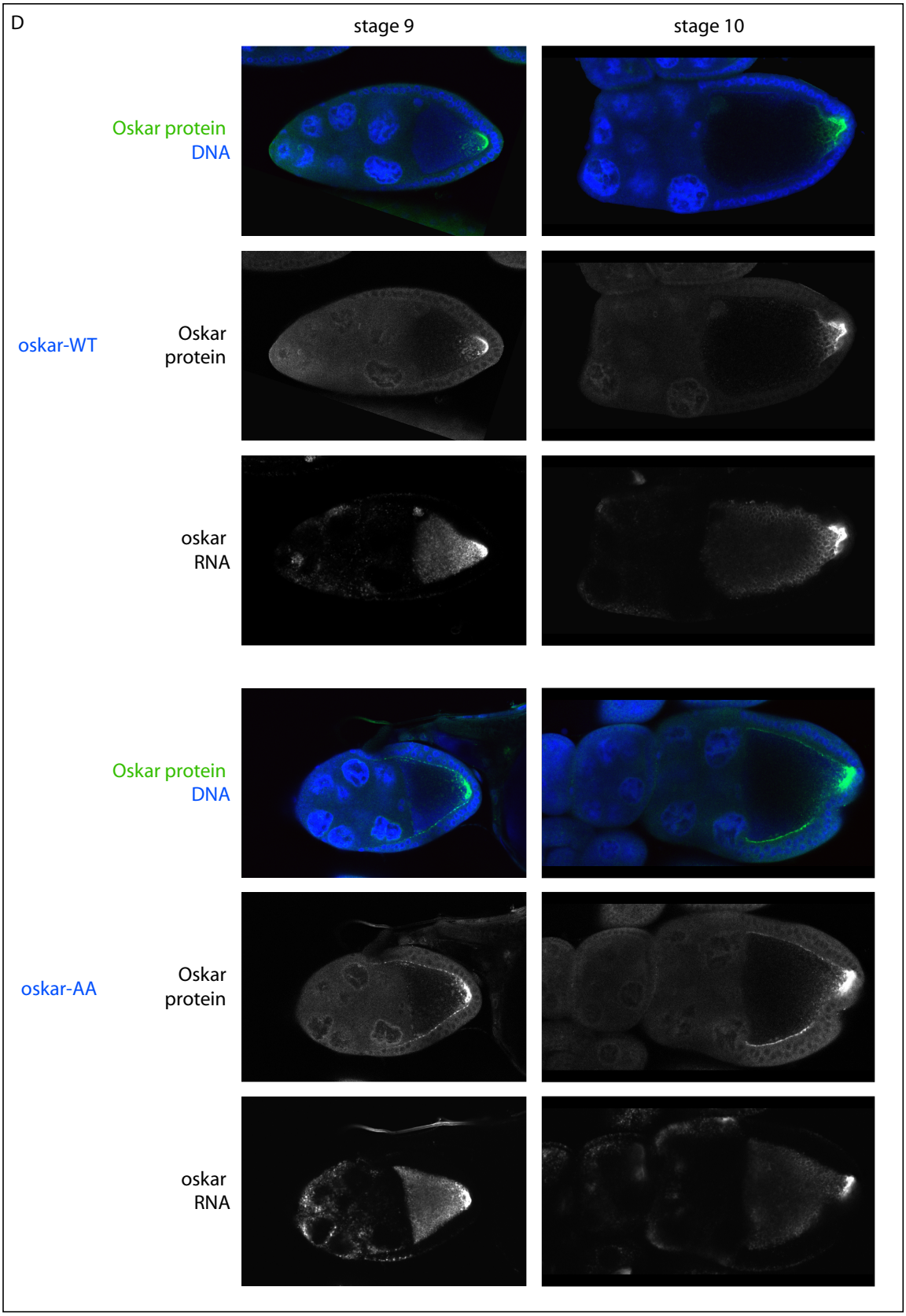
D
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Oskar protein
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3.4. Interaction of *oskar* molecules influences the levels of Oskar protein

in vitro dimerisation, *in vivo* hitch-hiking to the posterior pole and translational control all involve the loop of the dimerisation domain in the *oskar* 3'UTR. I therefore hypothesised that RNA-RNA interaction might directly control translation of Oskar.

To test this I restored RNA-RNA interaction by co-expressing *oskar*-AA with either *gfp-oskar* 3'UTR-WT or *gfp-oskar* 3'UTR-UU in *oskar* RNA null flies as already described in section 3.2.7 and depicted in Figure 22. I then analysed whether restoring RNA-RNA interaction could directly affect translation of these mRNAs.

In vivo oskar 3'UTR-WT hitch-hikes in only 16%, while the compensatory mutated *oskar* 3'UTR-UU accumulates posteriorly in 91% of egg chambers (see section 3.2.7). Consequently, if *oskar* interaction and translational control are indeed linked, when co-expressed, the compensatory mutated *oskar* 3'UTR-UU should rescue the translation defects of *oskar*-AA more efficiently than the wild-type and thus non-compensatory *oskar* 3'UTR-WT. *oskar*-AA and *gfp-oskar* 3'UTR encode proteins, Oskar and GFP, respectively. Thus, if RNA-RNA interaction indeed influences translation, both Oskar and GFP translation could potentially be affected. Therefore the Oskar and GFP protein levels were analysed for each genotype.

3.4.1. GFP protein levels

To test whether GFP expression was affected by co-expressing either *gfp-oskar* 3'UTR-UU or *gfp-oskar* 3'UTR-WT with *oskar*-AA, ovaries were dissected and the GFP signal in oogenesis analysed. While both *gfp-oskar* 3'UTR -WT and -UU are strongly expressed in the unfertilised egg, neither produces a detectable GFP signal in early- and mid-oogenesis (Figure 23). To control that the GFP signal was not simply lost due to too stringent fixation conditions, PTB-GFP was analysed as a positive control and showed a clear GFP signal in the nuclei of nurse cells and at the posterior pole of the oocyte (Figure 23).

By western blotting of ovarian extracts from flies co-expressing *oskar*-AA with either *gfp-oskar* 3'UTR-UU or with *gfp-oskar* 3'UTR-WT, GFP protein was detected, but no differences in protein levels between the co-expressing lines was observed (data not shown). The GFP protein detected is most likely due to the presence of young, unfertilised eggs that remained associated with the ovaries in the ovarian extract preparations.

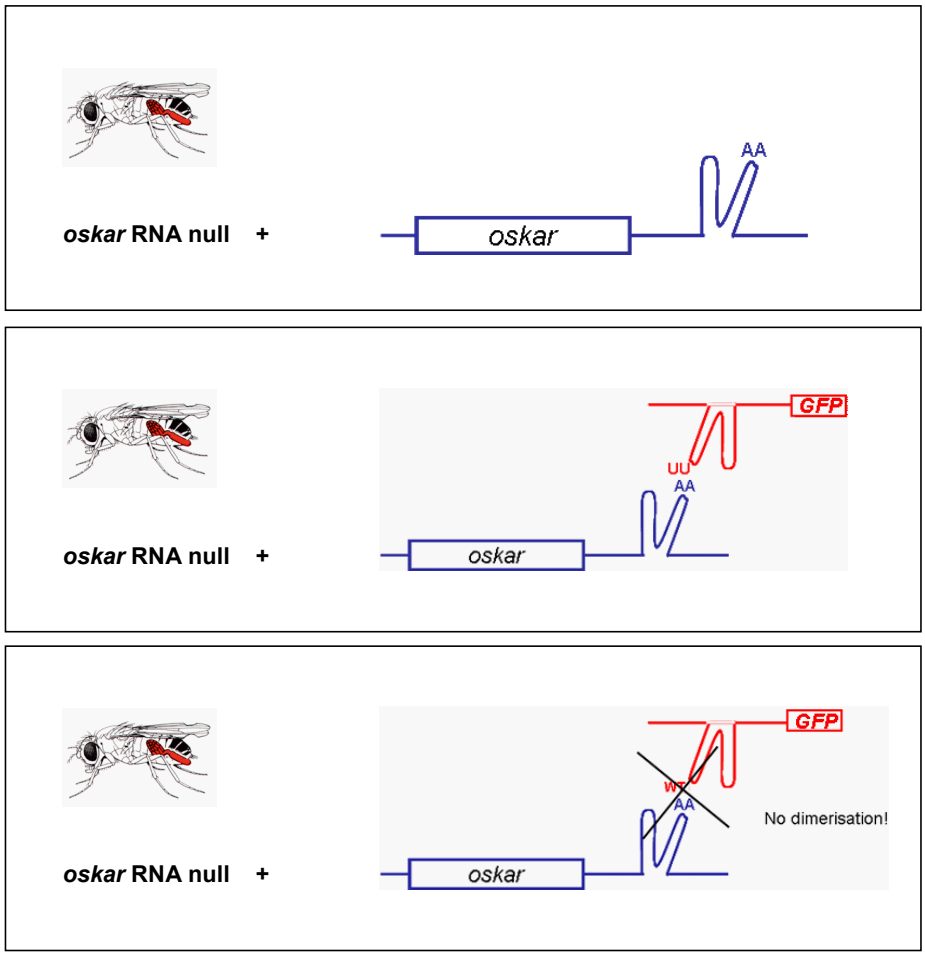

Figure 22: Schematic of experiments performed in section 3.4.
oskar-AA (in blue) was expressed alone, or co-expressed with either *gfp-oskar* 3'UTR-WT or -UU (in red). I then analysed if the presence of either of the *gfp-oskar* 3'UTR RNAs influences the expression levels of Oskar protein produced from the *oskar-AA* transgene. I also analysed whether the GFP protein is differentially expressed from *gfp-oskar* 3'UTR RNAs in the presence and absence of *oskar-AA* mRNA.

In summary, GFP protein was not expressed well enough to determine whether RNA-RNA interaction might influence translation of the *gfp-oskar* 3'UTR RNAs. It appears that GFP is not expressed from the gfp-oskar 3'UTR until late oogenesis. Only when removing Bruno response elements from the *gfp-oskar* 3'UTR, could GFP be expressed in young stages (data not shown). Furthermore, it was previously shown that sequences at the 5'end of the *oskar* RNA are required to overcome Bruno-mediated repression while the RNA is unlocalised during early oogenesis and at stage 9, once the RNA is localised at the posterior pole. Hence the lack of expression of GFP is probably due to the combined presence of binding sites for the translational repressor Bruno and absence of the *oskar* 5'UTR translation derepression sequences (Kim-Ha et al. 1995; Gunkel et al. 1998).

3.4.2. Oskar protein levels

I next analysed the levels of Oskar protein translated from *oskar*-AA co-expressed with either *gfp-oskar* 3'UTR-UU or *gfp-oskar* 3'UTR-WT. First the embryo hatching rates were determined. Neither co-expression of *gfp-oskar* 3'UTR-WT or *gfp-oskar* 3'UTR-UU with *oskar*-AA lead to an increase in the hatch-rate of embryos above what I observed for *oskar*-AA alone (Figure 24). Interestingly though, the developmental defects preventing the hatching of embryos were found to be quite different for each genotype. As expected from the invariant hatching rates, the frequency of embryos showing wild-type denticle belts remained un-changed for all genotypes.

Females expressing *oskar*-AA alone produced embryos, 89% of which displayed anterior patterning defects due to Oskar protein over-expression, 76% with very strong defects (Figure 25). Co-expression of *oskar*-AA with *gfp-oskar* 3'UTR-WT resulted in a slight reduction (to 85%) in the percentage of anterior defective embryos, with 68% showing very strong defects (Figure 25). However, when *oskar*-AA was co-expressed with the compensatory mutated *gfp-oskar* 3'UTR-UU, only 60% of embryos displayed anterior malformations, 44% showing very strong defects (Figure 25).

Thus, co-expression of the compensatory mutated *gfp-oskar* 3'UTR-UU with *oskar*-AA leads to a strong reduction of Oskar over-expression phenotypes in embryogenesis. For comparison, when expressing *oskar*-WT RNA at a similar high level, 50% of the embryos showed anterior patterning defects (data not shown). A reduction of anterior defects from 89% when *oskar*-AA is expressed alone to 60% when *oskar*-AA is co-expressed with *gfp-oskar* 3'UTR-UU therefore corresponds to a 75% rescue of the *oskar*-AA RNA caused phenotype.

A small but reproducible reduction in Oskar protein amount could also be detected by western blotting of ovarian extract. However here, I could not observe differences in Oskar protein levels when co-expressing either -WT or -UU *gfp-oskar* 3'UTR with *oskar*-AA (Figure 26). Both isoforms of Oskar, Long and Short, were reduced to the same extent, showing that the mechanism reducing translation does not specifically affect expression of one of the two isoforms.

Hence, the levels of Oskar protein expressed from *oskar*-AA can be influenced by co-expressing *gfp-oskar* 3'UTR-WT RNA, and more strongly by co-expressing the compensatory mutated *gfp-oskar* 3'UTR-UU RNA. As already shown in section 3.2.7, both analysed *oskar* 3'UTRs were expressed equally and enriched similarly in young oocytes, excluding the possibility that the differences in Oskar protein levels are caused by different *gfp-oskar* 3'UTR expression levels.

In order to have nearly one hundred percent anterior defects when starting the experiment, for all experiments presented I used a line (#18) expressing high levels of *oskar*-AA RNA. The Oskar protein levels expressed from the low expressing *oskar*-AA line (#19) could also be reduced when co-expressing the compensatory mutated 3'UTR (data not shown).

Taken together these data therefore indicate that the compensatory mutated *gfp-oskar* 3'UTR-UU expressed in trans can strongly influence Oskar protein levels expressed from an *oskar*-AA RNA.

Both *gfp-oskar* 3'UTRs were shown to interact with *oskar*-AA in the *in vivo* hitch-hiking assay and to influence Oskar protein levels expressed from *oskar*-AA RNA and in the two experiments the compensatory mutated *gfp-oskar* 3'UTR-UU RNA could hitch-hike with *oskar*-AA better and stronger repress *oskar*-AA translation. This most likely reflects the ability of either *gfp-oskar* 3'UTR to partially, and of *gfp-oskar* 3'UTR-UU to very strongly interact with *oskar*-AA via the dimerisation domain.

Thus, I would like to propose a mechanism of translational control based on direct RNA-RNA interaction, which to my knowledge has not been described previously for any other RNA. This novel mechanism highlights the fact that *oskar* translation is regulated by several mechanisms acting additively, presumably in order to tightly regulate Oskar protein expression during oogenesis. In conclusion, these findings indicate that direct RNA-RNA interaction is required for translational repression of *oskar* mRNA prior to its localisation at the posterior pole.

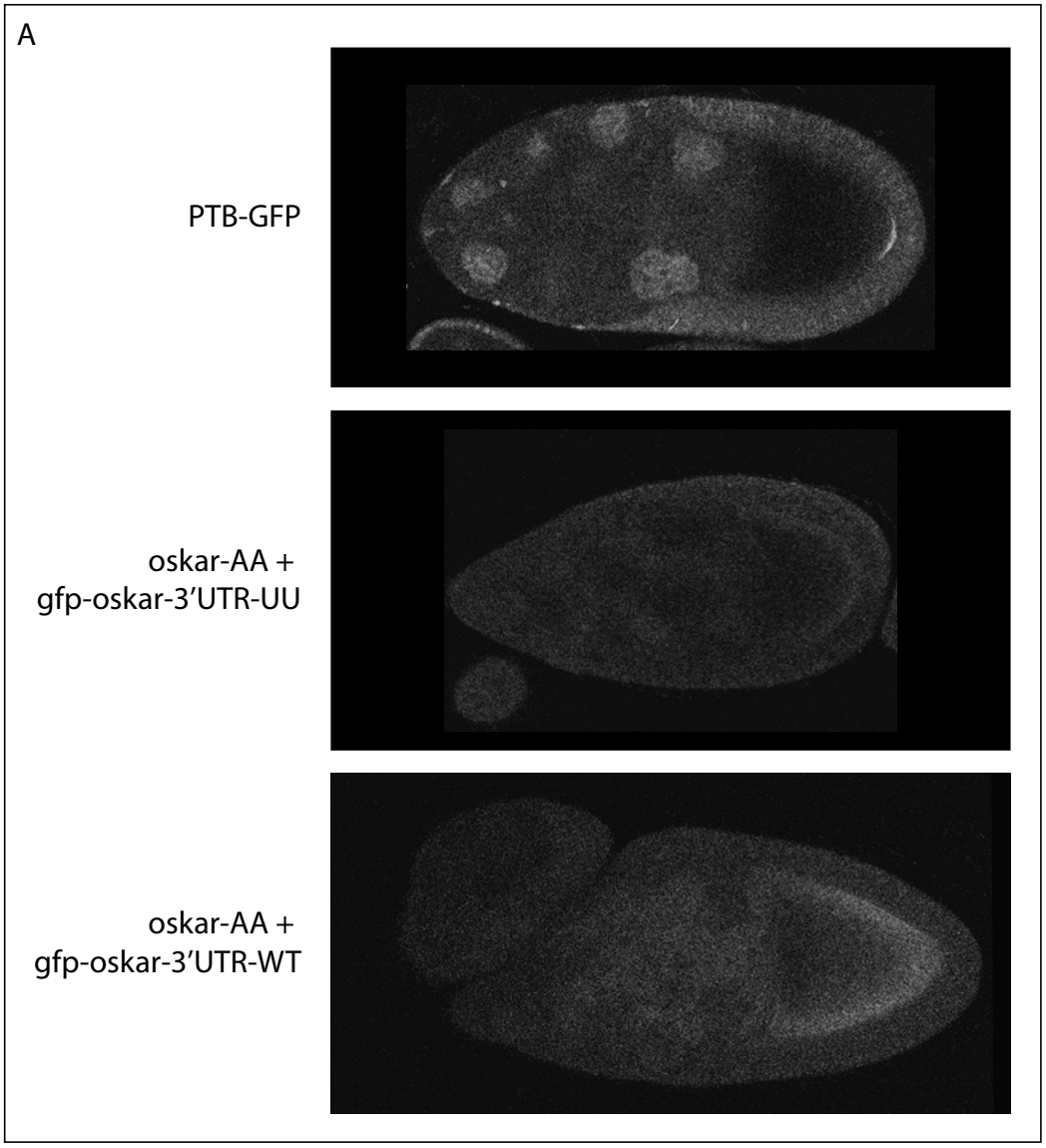

Figure 23: GFP is not expressed from the *gfp-oskar* 3'UTR RNAs.
No GFP signal was detected in stage 9 egg chambers that co-expressed either *gfp-oskar* 3'UTR-WT or- UU with the *oskar-AA*. In contrast, posterior GFP was observed in egg-chambers expressing PTB-GFP as a positive control.

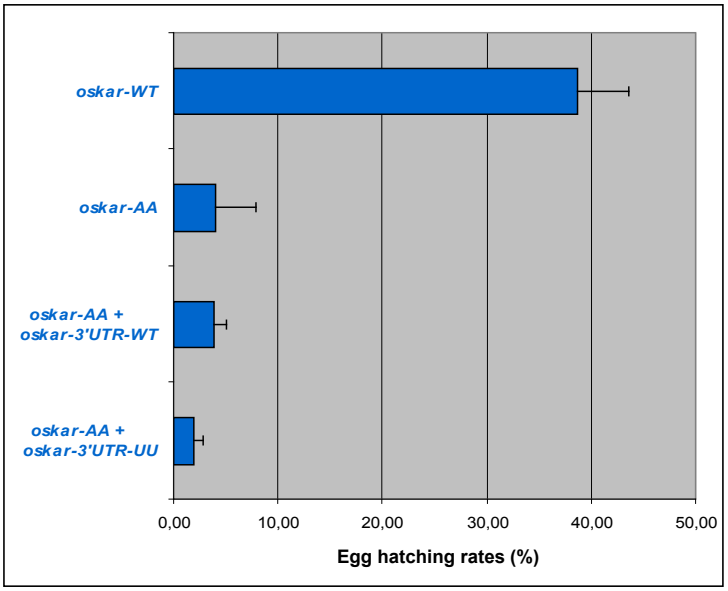

Figure 24: The lethality of *oskar*-AA embryos was unchanged by *gfp-oskar* 3'UTR co-expression.

The hatching rate of embryos produced by *oskar-AA*-expressing flies was compared to that of embryos produced by flies co-expressing *oskar-AA* and either *gfp-oska*r 3'UTR-WT or -UU. Neither WT nor the –UU 3'UTR RNA co-expression positively affected the survival of embryos. For comparison, I also analysed embryos produced by *oskar-WT* flies.

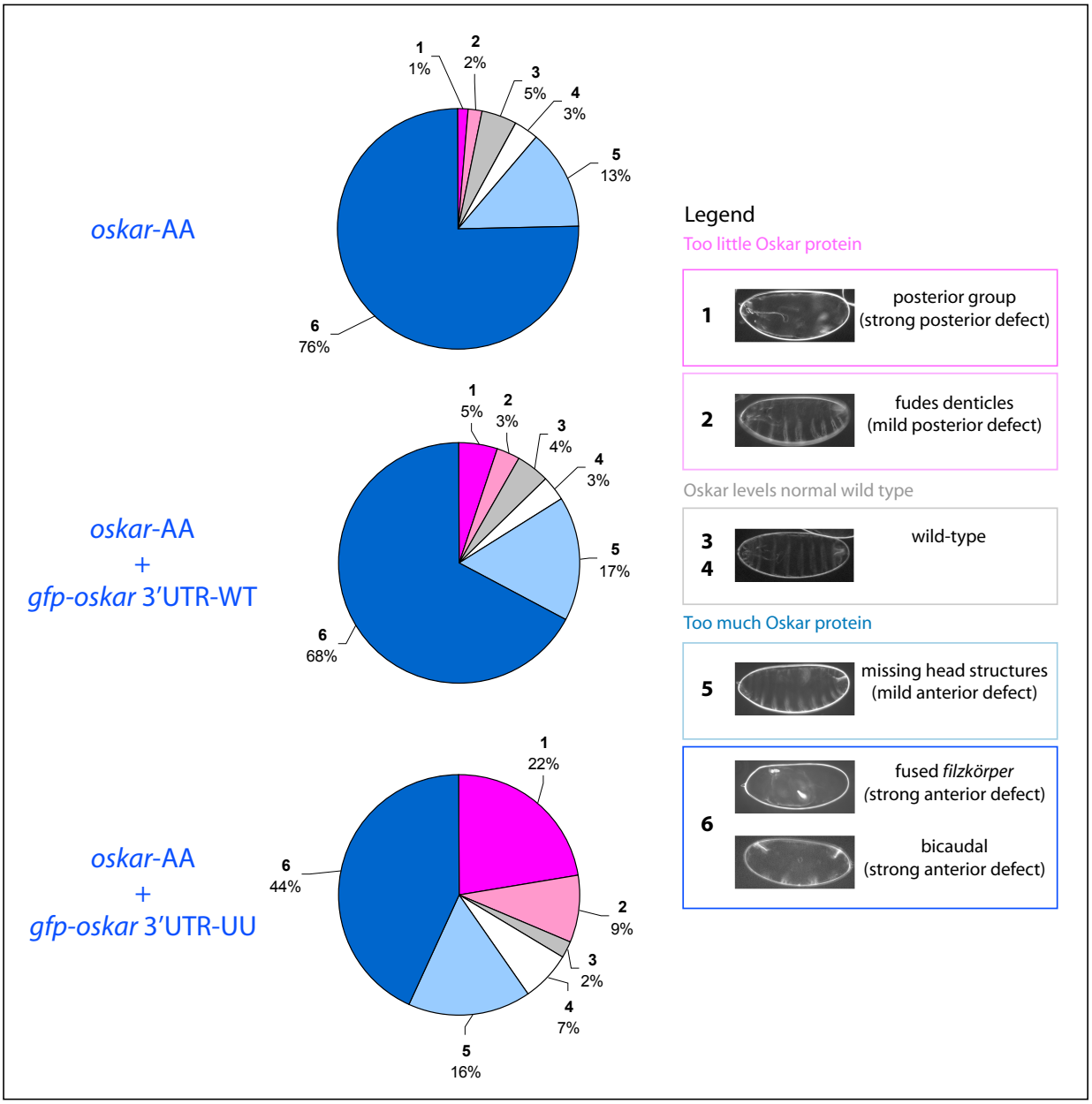

Figure 25: The spectrum of embryonic defects is altered when *oskar-AA* and *gfp-oskar* 3'UTR were co-expressed.
Co-expression of *oskar-AA* and the wild-type *oskar* 3'UTR mildly influences embryonic patterning. Co-expression of the compensatory *oskar*-UU 3'UTR strongly alters the embryonic patterning defects produced by *oskar*-AA. The following phenotypes were scored: mild and strong "posterior group" phenotypes (pink), mild anterior head defects (pale blue) and "bicaudal" embryos (dark blue). The wild-type embryos were scored as hatched (white) or un-hatched (grey).

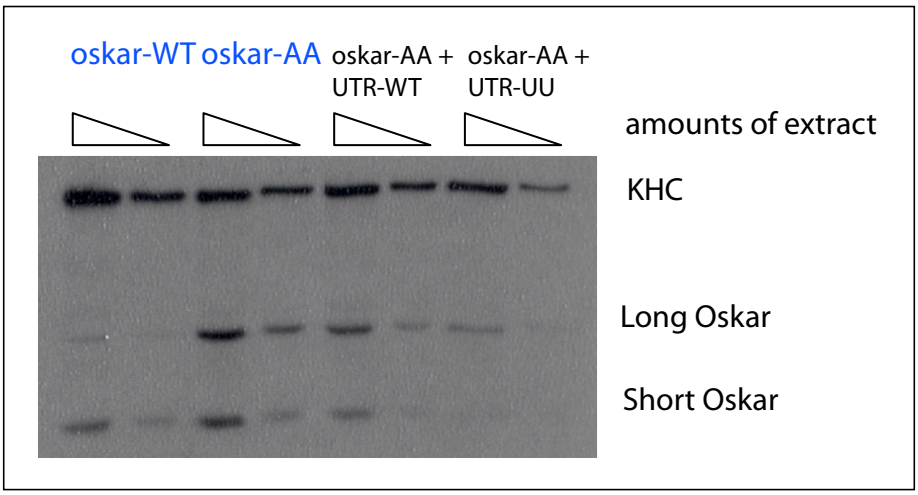

Figure 26: Oskar protein levels are reduced when *oskar-AA* and *oskar 3*'UTR are co-expressed.
Western blot showing that the levels of both Oskar isoforms are reduced in ovarian protein extracts from flies co-expressing *oskar*-AA with either *gfp-oskar* 3'UTR-WT or –UU, compared to flies expressing oskar-AA alone. Different amounts of egg-chamber extract were loaded and the blot probed with anti-Oskar antibody; anti-Kinesin heavy chain antibody was used in parallel as a loading control.

4. Discussion: Part I

oskar reporter RNAs can indirectly accumulate at the posterior pole dependent on the presence of endogenous *oskar* mRNA, a process described as hitch-hiking (Hachet and Ephrussi 2004). Here I have presented evidence that the *oskar* 3'UTR reporter RNA directly interacts with endogenous *oskar* mRNA in this process. Furthermore, I have shown that hitch-hiking of *oskar* RNA involves a conserved domain that *in vitro* permits RNA dimerisation. *In vivo,* RNA interaction via the dimerisation domain is critical for *oskar* mRNA translational repression. The present study therefore provides the first evidence that mRNA-mRNA interactions *in trans* can promote translation repression in eukaryotes.

4.1. oskar RNA molecules interact via their dimerisation domains

I have shown that intronless mRNAs with mutations in the dimerisation domain loop of the *oskar* 3'UTR are less competent than wild-type *oskar* 3'UTR reporters to accumulate at the posterior pole by hitch-hiking. The co-expression of an *oskar* RNA molecule bearing compensatory mutations restored posterior accumulation of the reporter 3'UTR. I conclude that the dimerisation domain is involved in hitch-hiking of *oskar* 3'UTR reporters on endogenous *oskar* molecules and that this hitch-hiking requires direct interaction of *oskar* RNA molecules in *trans*. This is in agreement with the finding of (Hachet and Ephrussi 2004) that the *oskar* 3'UTR promotes hitch-hiking, and favours a proposed model that this hitch-hiking involves a direct RNA-RNA interaction (Hachet and Ephrussi 2004). A direct RNA

contact via the 3'UTR was previously shown *in vitro* for the maternal mRNA *bicoid* that involves the formation of an a double loop-loop interaction (Ferrandon et al. 1997; Wagner et al. 2001), while *oskar* dimerisation depends on a "kissing"-loop mechanism (Dr Christine Brunel).

Hachet and Ephrussi alternatively hypothesised that hitch-hiking might involve protein-protein interaction. For example Bruno protein can directly interact with binding sites in the *oskar* 3'UTR and has been shown to induce formation of heavy particles and RNA oligomerisation (Kim-Ha et al. 1995; Chekulaeva et al. 2006). Thus it is conceivable that hitch-hiking involves both RNA-RNA interactions via the dimerisation domain, as well as proteins. A third possibility would be that hitchhiking could also be mediated by another RNA molecule. While I cannot exclude the possibility that proteins and other RNAs are also necessary for hitch-hiking of *oskar*, the analysis of compensatory mutations argues in favour of a direct RNA-RNA interaction via the dimerisation domain loop.

As mentioned above, substitutions in the dimerisation domain loop reduce, but do not completely abrogate posterior localisation of an *oskar* 3'UTR RNA at stage 9 of oogenesis. I also observed that deleting the loop and the distal stem of SLII (*oskar* 3'UTR-ΔSLIId) still allows hitch-hiking in a fraction of egg chambers, and that the SLI-II region alone is not sufficient to enrich at the posterior pole of the oocyte along with localisable *oskar* mRNA. Thus, a second conclusion is that hitch-hiking requires additional *oskar* 3'UTR sequences, outside the dimerisation domain (SLII). Indeed, to date, I could not observe any hitch-hiking when I deleted sequences either upstream or downstream of the SLI-II region in *oskar* 3'UTR reporters (data not shown). This is also consistent with data from Kim-Ha et al., who analysed localisation of *lacZ-oskar* 3'UTR chimeric RNAs in otherwise wild-type flies, with the intention of mapping the signals required for posterior *oskar* RNA accumulation. We now know that what they actually observed was hitch-hiking of the chimeric RNA along with the endogenous *oskar* mRNA. Kim-Ha et al showed that deletions both at the 5' and at the 3' end of the *oskar* 3'UTR abrogated posterior RNA accumulation (Kim-Ha et al. 1993). In summary, the observations above suggest that the integrity of the *oskar* 3'UTR might be necessary for posterior hitch-hiking. At least two, not mutually exclusive scenarios are conceivable: either hitch-hiking involves a bi- or multi-partite signal within the *oskar* 3'UTR, or hitch-hiking is a cooperative action between the dimerisation domain and proteins. In the latter case, proteins binding to *oskar* 3'UTR RNA could strengthen an initial RNA dimerisation event, or conversely, RNA-RNA contact could reinforce the binding of proteins to *oskar* 3'UTR RNA. The *oskar* 3'UTR actually does contain a second, highly conserved

region upstream of the dimerisation domain that, according to *mfold* predictions, folds into a stem-loop resembling the dimerisation domain.

Dimerisation of RNA molecules is not widely studied, and is best characterised in the case of retroviral genomic RNAs that form tight dimers in the virion. Dimerisation of *HIV-1* is initiated by a "kissing"-loop interaction of two *HIV-1* RNA monomers via a dimerisation initiation sequence (DIS; (Clever et al. 1996). In a second step an extended RNA duplex of two *HIV-1* RNAs is formed (see also Figure 27A). This dimer is then stabilised by the Gag protein that "zippers" the dimerisation along the RNA (Russell et al. 2004; Lever 2007). It is interesting that the RNA-RNA interactions of *oskar* and *HIV-1* involve the same "kissing loop" mechanism via a palindromic, GC-rich sequence of 6 nucleotides (*oskar*: cc**gc**gg; *HIV1*: gc**gc**gc; *SIV*: gt**gc**ac; (Clever et al. 1996). Direct RNA-RNA contacts could also be relevant for oligomerisation of co-transported mRNAs that localise to specific subcellular sites via the same transport pathways. For example, αCaMKII, ARC and NG mRNAs co-localise in hippocampal neurons and in granules along neurites and *IST2* and *ASH1* are co-transported into the bud of yeast cells. In both cases, it is unclear, apart from hnRNP A2 binding, what triggers this RNA copackaging in transport RNPs (Gao et al. 2008; Lange et al. 2008).

In summary, similar to the *HIV-1* scenario, *oskar* mRNA dimerisation could be initiated by a direct RNA-RNA interaction and subsequently strengthened by RNA binding proteins, for example Bruno. Although more research is needed to identify all *cis*-regulatory sequences necessary for localisation of *oskar* 3'UTR reporters, I could show that the dimerisation domain is indeed involved in *oskar* RNA-RNA interactions during hitch-hiking. Thus, it is now established that the hitch-hiking assay can reveal the *in vivo* strength of the interaction between *oskar* mRNA and *oskar* 3'UTR reporters.

4.2. Hitch-hiking of *oskar* mRNA

The ability of *oskar* 3'UTR reporters to localise indirectly at the posterior pole of the oocyte was termed hitch-hiking. During the course of my research, I characterised *cis*-regulatory sequences necessary for this process. When does hitch-hiking occur during oogenesis? I observed that mutations in the dimerisation domain strongly affect posterior enrichment of reporter *oskar* 3'UTR RNA at stage 9, but to a lesser degree at stage 10. Several models could explain this finding. Hitch-hiking-based localisation of *oskar* 3'UTR RNA could occur continuously at both stages 9 and 10 via the same *cis*-regulatory signals. In this case, mutations in the dimerisation domain could slow hitch-hiking at stage 9 and lead to a delayed

posterior enrichment that is nevertheless almost indistinguishable from that wild-type *oskar* 3'UTR reporters at stage 10. Mutating all necessary *cis*-regulatory elements in the *oskar* 3'UTR might completely abolish hitch-hiking in both stage 9 and 10 oocytes. While the majority of endogenous *oskar* mRNA is localised by stage 10A, injected *oskar* mRNA can accumulate at the posterior pole as late as stage 11, suggesting that some transport to the posterior is ongoing at that stage (Glotzer et al. 1997). Hence, the *oskar* 3'UTR reporters may hitch-hike at least until stage 11 of oogenesis.

Alternatively hitch-hiking could be a mechanism that applies exclusively to stage 9 localisation of *oskar* 3'UTR reporters, while localisation at stage 10 might depend on other *cis*-regulatory signals and distinct mechanism. According to the latter hypothesis, one would expect to find a minimal region of the *oskar* 3'UTR that would not enrich at stage 9, but, by the hypothetical second localisation mechanism, would fully enrich at the posterior pole at stage 10 of oogenesis. In late oogenesis, the nurse cells regress and expel their cytoplasm into the oocyte. Cytoplasmic streaming then creates a strong movement within the cell that quickly disperses the "dumped" contents within the oocyte (Gutzeit 1986; Theurkauf et al. 1992). However, slow ooplasmic streaming can be observed already at stages 9 and 10A of oogenesis (Gutzeit, 1986b). Thus, a mechanism such as cytoplasmic streaming might be responsible for the late accumulation of *oskar* 3'UTR reporters at the posterior pole, dependent on signals outside the dimerisation domain.

When during oogenesis is dimerisation of *oskar* mRNA and *oskar* 3'UTR reporters initiated? The interaction of these RNAs could take place in the nucleus or in the cytoplasm of nurse cells, at the anterior pole or at the posterior pole of the oocyte. All *oskar* 3'UTR reporters enrich similarly in young wild-type oocytes, and when they were expressed in *oskar* RNA null genetic backgrounds, in which hitch-hike along with endogenous *oskar* mRNA was no longer possible, the reporter RNA could still localise to the developing oocytes but not enrich at the posterior pole. While this does not exclude the possibility that *oskar* mRNA and the *oskar* 3'UTR reporters interact already in the nurse cells, it shows that this interaction is not a pre-requisite for entering the oocyte.

Interaction of *oskar* mRNA and *oskar* 3'UTR reporters might also occur both at the anterior of oocytes at stage 8/9 or at the posterior pole at stage 9. If the RNA interaction takes place at the anterior pole, *oskar* mRNA and *oskar* 3'UTR reporters should be co-transported to posterior pole. In contrast, if dimerisation of *oskar* molecules occurs only at the posterior pole, the RNA molecules should be found in different populations of transport particles and only co-localise at the posterior pole. In this case, hitch-hiking would be an RNA-based co-

anchoring mechanism. *oskar* mRNA anchoring at the posterior pole is necessary *in vivo* and requires Oskar protein, however, it is conceivable that also RNA-RNA interactions could additionally act to safely attach *oskar* at the posterior pole (Markussen et al. 1995; Rongo et al. 1995).

Two indications favour the model of a co-transport rather than a co-anchoring of *oskar* RNAs: first, I did not observe a delay between *oskar* mRNA and *oskar* 3'UTR reporters enriching at the posterior pole. If *oskar* mRNA would indeed anchor the *oskar* 3'UTR, it should reach the posterior pole at least slightly before the *oskar* 3'UTR does. In some cases the *oskar* 3'UTR RNA was localised at the posterior pole even before Oskar protein was detected, suggesting that there is very little or no delay between *oskar* mRNA and *oskar* 3'UTR accumulation. Secondly, non-hitch-hiking *oskar* 3'UTR reporter RNA was not dispersed throughout the oocyte, but was detected tightly localised at the anterior cortex. This indicates that reporter RNAs might not have been transported after stage 8. If indeed hitch-hiking were purely a co-anchoring mechanism, I would expect some RNA to be dispersed in the oocyte, on its way to the RNA anchor. It should be possible to test directly whether *oskar* mRNA and *oskar* 3'UTR reporter RNA are found in one particle or rather in separate particle populations during their transport to the posterior, for example by double *in situ* electron microscopy or by tracking of fluorescently tagged RNAs *in vivo*.

4.3. Control of *oskar* mRNA translation via the dimerisation domain

To analyse the role of the dimerisation domain in *oskar* mRNA regulation, I substituted two nucleotides of the dimerisation domain loop in the context of a genomic *oskar* mRNA (*oskar*-AA). I then expressed this transgenic mRNA in *oskar* RNA null oocytes. *oskar*-AA mRNA could rescue the early oogenesis arrest and localised as does *oskar* wild-type RNA, showing that the integrity of the dimerisation domain loop is not critical for these processes. Because the *oskar* 3'UTR reporters did show hitch-hiking defects, it was surprising that localisation of the genomic *oskar-AA* mRNA appeared normal. Two explanations are possible: first, *oskar* mRNA localisation could be independent of the dimerisation domain and hitch-hiking or, secondly, the dimerisation domain act redundantly with another, yet uncharacterised signal in the *oskar* 3'UTR, in posterior enrichment. So far, Hachet and Ephrussi described that splicing of intron 1 together with the *oskar* 3'UTR are necessary for *oskar* mRNA localisation, but the exact contributions of the 3'UTR were not previously investigated (Hachet and Ephrussi 2004). As mentioned above, mutations in the dimerisation domain reduce, but do not

completely abolish, posterior hitch-hiking, suggesting that also for this process multiple signals in the *oskar* 3'UTR are required. A third possibility is that mutation of the dimerisation domain does cause a mild defect or delay in *oskar* localisation that ultimately has no consequence for proper embryonic development. These hypothetical minor localisation defects might not be detectable by *in situ* hybridisation techniques, but could possibly be revealed by live imaging of RNA transport. In summary, the data presented here suggest that the dimerisation domain, and consequently hitch-hiking, does not play a critical role in *oskar* mRNA localisation.

While localisation of the *oskar*-AA mRNA is normal, translation of Oskar protein is highly mis-regulated. *oskar*-AA embryos display strong patterning defects and express unusually high levels of Oskar protein suggesting that the dimerisation domain is required for normal *oskar* translational control. At least three explanations for these observations are possible: firstly the *oskar*-AA mutation could affect a miRNA target site or secondly, the binding of an unknown translational repressor. Thirdly, RNA-RNA contact via the dimerisation domain might directly regulate translation.

We do not have evidence that translational regulation of *oskar* mRNA during oogenesis is regulated by a miRNA and several facts argue against such a scenario. None of the known *Drosophila* miRNAs (and piRNAs) are predicted to bind to the dimerisation domain of *oskar* mRNA (personal communications with Alexander Stark). Additionally, the dimerisation domain is found centrally within the *oskar* 3'UTR, a region that is rarely targeted by miRNAs. Target sites of miRNAs typically are not evenly distributed along 3'UTRs but peak near the polyadenylation site and proximal to the stop codon, while the central part of 3'UTRs are statistically depleted of miRNA binding sites (Majoros and Ohler 2007). The single predicted miRNA target site in the *oskar* 3'UTR is that of *miR*-6, which is located 418 nucleotides upstream of the dimerisation domain. *miR*-6 was shown to regulate degradation of maternal mRNAs at the onset of zygotic transcription in early embryogenesis (Bushati et al. 2008).

Alternatively, mutation of the binding site of a translation repressor would lead to ectopic *oskar* translation. When an *oskar* transgene with mutated Bruno response elements (BRE) was expressed in the germline, embryos derived from these mothers showed patterning defects similar to embryos expressing *oskar*-AA (Kim-Ha et al. 1995). However, as all BREs are fully intact in *oskar*-AA RNA. Instead other repressors might be hindered in their action. Several proteins are involved in regulation of Oskar expression, for example Hrp48 and PKA-R1, of which Hrp48 was shown to directly bind to *oskar* mRNA (Yano et al. 2004; Yoshida et

al. 2004). When I co-expressed a compensatory mutated *oskar* 3'UTR reporter and *oskar*-AA in the germline, I partially suppressed the de-repression of *oskar*-AA RNA. This "rescuing" effect of the compensatory mutations argues against the binding of a translational repressor and, together with the *in vitro* and *in vivo* data showing direct interaction of *oskar* 3'UTR RNAs, favours a model in which this RNA-RNA contact influences Oskar translation. A simple explanation for our findings therefore is that RNA interaction via the highly conserved stem-loop directly promotes translational repression of un-localised *oskar* mRNA.

Two protein isoforms, Long and Short Oskar, are encoded by *oskar* mRNA and expressed in a stereotyped ratio of approximately 1:4. In western blots, both protein isoforms are up-regulated when comparing *oskar*-AA to wild-type control extracts. In particular, Long Oskar is strongly over-expressed, resulting in a distorted ratio of ~1:1. The strong over-expression of Long and only mild over-expression of Short Oskar (the pole cell-inducing isoform), could explain the lack of anterior pole cells in the embryo of *oskar-AA* expressing flies. We have not investigated further whether the observed phenotypes are either due to a distorted protein ratio of Long and Short Oskar or alternatively are merely a consequence of the Oskar protein over-expression.

Thus, in summary, RNA-RNA interaction via the dimerisation domain in the *oskar* 3'UTR seems to promote translational repression of un-localised *oskar* mRNA, while it does not affect mRNA transport. It would be very appealing to investigate in greater detail the relationship between RNA interaction and translational control. One could try to re-establish translational silencing by substituting the dimerisation domain loop with the loop of another RNA that interacts by a similar "kissing"-loop mechanism, for example the *HIV-1* dimerisation initiation signal (DIS). However the *HIV-1* DIS and the *oskar* dimerisation domain loop differ only in two of six nucleotides, which might be too similar to draw meaningful conclusions. Alternatively the simian immunodeficiency virus (SIV) DIS, which differs in four out of six nucleotides but is less well characterised, could be tested (see above; (Clever et al. 1996). Apart from these, no further "kissing"-loop interactions have been described. Before analysing these loops in vivo, a thorough *in vitro* characterisation would be necessary.

4.4. Translational Control by RNA-interaction

oskar RNA molecules can engage in intermolecular RNA-RNA interactions that negatively influence their translation, a finding that to my knowledge has not been reported for any other eukaryotic mRNA. Prokaryotic plasmids, bacteriophages and transposons encode antisense RNAs that bind to complementary regions of target mRNAs. These 90 to 140 nucleotide long antisense RNAs (asRNA) are typically *cis*-encoded, i.e. on the reverse strand of the DNA, thereby allowing 100% complementarity over their entire length, and targeting a single RNA. In bacteria, examples of *trans*-encoded asRNAs are also known that are complementary only over a few base pairs and therefore have multiple targets RNAs. asRNAs can activate, but most often repress translation and typically act on the 5'UTR of their targets to ensure a prompt effect in the bacterial cell, where transcription and translation are coupled processes (Altuvia and Wagner 2000). The initial interaction of an antisense RNA with its target RNA is often established by a "kissing" interaction via GC-rich loops that subsequently induces the formation of a stable complex by propagation of base pairing and formation of a complex structure (**Error! Reference source not found.**; Simons and Kleckner 1988; Brantl 2002). For example OxyS interaction covers the Shine-Dalgarno sequence of *fhlA* mRNA and thereby prevents recruitment of the 30S initiation complex (Altuvia et al. 1998).

In eukaryotic cells, many examples for critical RNA-RNA interactions are also known, for example during splicing, RNA editing (plants) and translation (tRNAs). Eukaryotic mRNAs can also base pair with the 18S rRNA, an interaction that positively influences translation for example of the mammalian *Gtx* and *FGF2* mRNAs and requires formation of an extended duplex with a 9-nucleotide signal in the 5'UTR (Panopoulos and Mauro 2008). Short RNAs that regulate translation similar to bacterial asRNAs have also been described and are used in experimental biology for example as morpholinos and siRNAs (Green et al. 1986; Eisen and Smith 2008).

miRNAs, RNAs of a novel class that have been intensively investigated recently, base pair with their target mRNA to control gene expression. These ~21-nucleotide short RNAs typically bind the 3'UTR of mRNAs by forming an extended RNA duplex. miRNAs often have only imperfect complementarity with their target and can thereby affect several RNAs simultaneously (Jackson and Standart 2007; Filipowicz et al. 2008). In most cases, miRNAs silence translation, yet also degradation of the target mRNAs by deadenylation has been described (Jackson and Standart 2007). Translational repression induced by a miRNAs can occur by several mechanisms, for example by inhibition of translation initiation, defective elongation and premature termination (Eulalio et al. 2008). Recently it was shown that

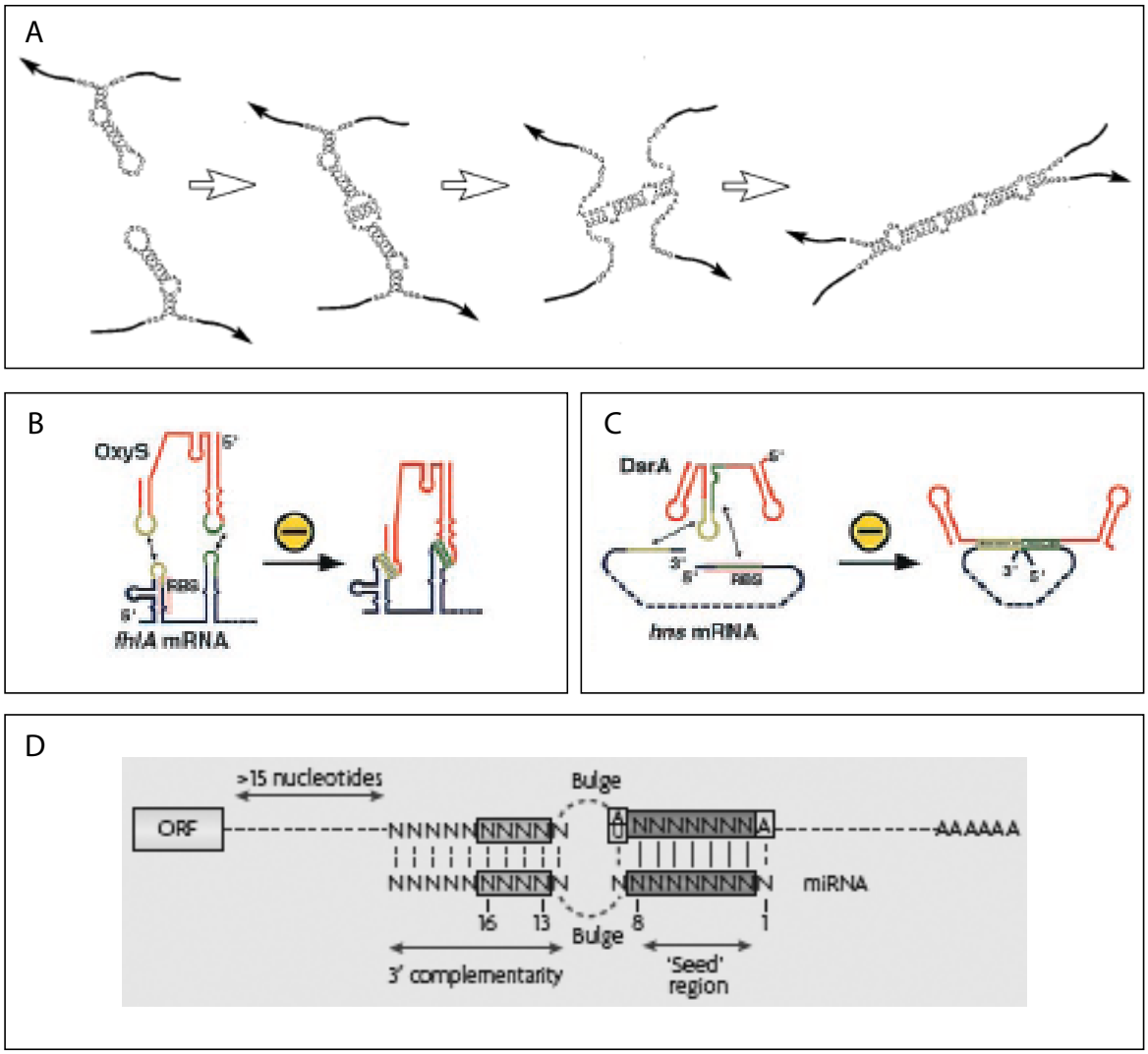

Figure 27: Examples of RNA-RNA interactions.
A. Initiation of HIV-1 RNA dimerisation via the DIS domain and subsequent formation of an extended duplex (from Clever et al., 1996). B, C. Two examples of bacterial asRNAs interacting with their target RNAs (from Altuvia and Wagner, 2000). D. Eukaryotic miRNA forming an extended duplex with its target mRNA (from Filipowicz et al., 2008).

translation of a reporter mRNA containing multiple miRNA target sites in its 3'UTR was repressed in a cap-dependent manner, while the same reporter could be translated from an IRES element (Pillai et al. 2005). This long-range action of 3'UTR bound miRNAs could be mediated by AGO proteins that show some sequence homology to the translation initiation factor eIF4E and therefore could compete for binding to the cap (Kiriakidou et al. 2007).

miRNAs were also found to accumulate in cytoplasmic foci referred to as P-bodies. These P-bodies are defined by the presence of certain proteins, of which the *D.melanogaster* homologues are Cup, Me31B and Dcp1, all also involved in *oskar* mRNA regulation (Keyes and Spradling 1997; Nakamura et al. 2001; Wilhelm et al. 2003; Lin et al. 2006). Although often implicated in mRNA degradation, P-bodies also reversibly sequester mRNAs from the translation pool as shown in yeast cells and their integrity requires RNAs (Brengues et al. 2005; Teixeira et al. 2005; Jackson and Standart 2007; Eulalio et al. 2008).

As the discovery of eukaryotic regulatory RNAs is still quite recent, it would be premature to assign all examples of translational control by RNAs to miRNAs. The control of *oskar* mRNA translation by RNA-RNA interaction via the 3'UTR might be representative of a novel mechanism whereby mRNAs can regulate their own translation.

4.5.A Model For *oskar* RNA-RNA interaction controlling translation

oskar RNA molecules directly interact via their dimerisation domain as observed *in vitro* and suggested by the *in vivo* data I have presented. This interaction might be initiated at the anterior of stage 8 egg chambers. Dimerisation then would mediate translational repression both of *oskar* molecules that are being transported to the posterior pole and of molecules that escaped from the transport machinery (Figure 28A). Consequently, if this interaction were disturbed, precocious *oskar* translation would occur from stage 8 onwards in the oocyte. Translation of this pool of un-localised *oskar* RNA might be continued throughout oogenesis, leading to high levels of ectopic Oskar activity. The ectopic Oskar protein would then cause formation of embryos with duplicated posterior structures that would arrest development prematurely (Figure 28B).

How can RNA-RNA interaction between *oskar* molecules promote translational silencing? Similar to bacterial asRNAs and the action of some eukaryotic miRNAs, this RNA interaction could interfere with translation initiation. Dimerisation via the *oskar* 3'UTR could for example induce a conformational change in the mRNA. Alternatively, in a manner similar to Bruno protein, which binds to sequences in the 3'UTR and then, via interaction with the

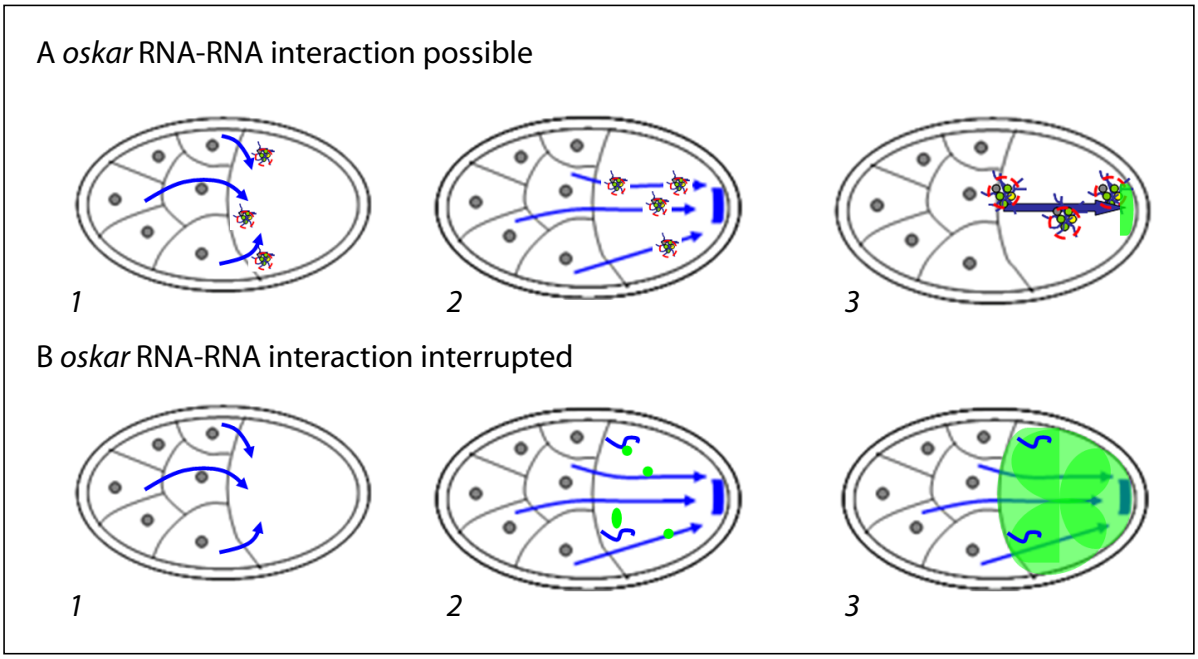

Figure 28: Model for *oskar* RNA interaction and translational control

A. *oskar* molecules can form RNA-RNA interactions.

1 - *oskar* RNAs interact.

2 - interacting RNAs are transported in RNP complexes.

3 - Oskar protein is translated at the posterior pole of the oocyte.

B. *oskar* RNA-RNA interaction is interrupted

1 - *oskar* molecules do not interact.

2 - *oskar* molecules localise, but are accessible to the translation machinery before, during and after transport.

3 – Oskar protein is dispersed throughout the whole egg-chamber.

eIF4E binding protein Cup, represses translation (Kim-Ha et al. 1995; Wilhelm et al. 2000; Nakamura et al. 2004; Chekulaeva et al. 2006), this new structure might allow recruitment to the RNA of an eIF4E competitor that interacts with the cap, thereby inhibiting recruitment of eIF4G. It is also possible that the induced RNA conformational change would prevent binding of a translation-promoting protein, such as the *D.melanogaster* PABP, which is required for proper Oskar protein expression (Castagnetti and Ephrussi 2003). Another possibility is that the dimerised RNA interacts directly with the 5'UTR and thereby sterically inhibits either initiation or elongation. Although involved in cup-independent translation initiation, "kissing"-loop interactions between the 5' and 3'UTR were shown to occur in uncapped, non-polyadenylated viral RNAs (Rakotondrafara and Miller 2008).

Of course, it is also possible that a fully dimerised *oskar* RNA simply occludes the binding of translation initiation complexes, similar to the dimerised *HIV-1* genomic RNA. Finally, analogous to P-bodies in yeast or the action of miR-2 in *D.melanogaster*, interacting *oskar* mRNAs could be sequestered from the cytoplasm into heavy silencing particles and thus remain inaccessible to the translation machinery. Translationally repressed *oskar* RNA is found in heavy particles that are that are dependent on Bruno protein. Bruno can promote RNA oligomerisation of a short fragment of the *oskar* 3'UTR; however, *oskar* containing mutated BREs can hitch-hike to the posterior pole (Kim-Ha et al. 1995; Chekulaeva et al. 2006); Chekulaeva, unpublished results). It is therefore tempting to speculate that *oskar* RNA interactions, in concert with Bruno-mediated oligomerisation and/or other proteins, initiate the sequestering un-localised *oskar* mRNA into particles that are inaccessible to ribosomes and thereby repress translation.

4.6. Open questions

In summary, I have shown that distinct *oskar* RNA molecules interact directly and that this interaction is required for maintenance of un-localised *oskar* mRNA in a translationally repressed state. To further understand translational control of *oskar* mRNAs by RNA-RNA interaction, it would be of interest to first clearly define the different RNA regions involved in this interaction and secondly to determine the contribution of each to *oskar* regulation. The mapping of further *oskar* RNA regions required for RNA interaction could be done by assaying hitch-hiking at stage 9 and simultaneously assessing the translation state of the RNA. Unfortunately the existing *gfp-oskar* 3'UTR reporter is not useful to investigate the link between hitch-hiking and translation, as it does not contain the 5' region of *oskar* mRNA

implicated in translational activation (Gunkel et al. 1998). Therefore de-repression of the *gfp-oskar* 3'UTR reporter cannot faithfully predict the regulation of *oskar* mRNA translation. As a further proof of the apparent link between RNA interaction and translation, it would be nice to test if restoring RNA interaction by replacing the *oskar* dimerisation domain with a heterologous palindrome can restore normal translational control.

What has not been addressed here but is potentially quite interesting is how at the posterior pole *oskar* mRNA translation is activated. Are *oskar* mRNAs dimerised at the posterior pole? If so, how is this interaction, which is mediated by the dimerisation domain, alleviated? Are proteins involved and where do they bind? Another interesting issue is how the ratio of Long and Short Oskar is produced, and whether this is influenced by RNA dimerisation.

A conclusion from this analysis is that *oskar* mRNA regulation is controlled by several mechanisms that might act cumulatively. In the future, it will be very interesting to investigate whether this also is the case for other mRNAs that are translationally silenced during transport.

More generally, the link of dimerisation and translation found for *oskar* mRNA could also be important for other interacting RNAs. For example, it is tempting to speculate that both *oskar* mRNA and retroviruses could use dimerisation to become inaccessible to the translation machinery. How base-pairing contributes to silencing of other RNAs that interact with and repress their target mRNA remains to be addressed.

4.7.Epilogue

It is intriguing that *oskar* is the first eukaryotic mRNA described to control its own translation by RNA dimerisation. All other cases where RNA interaction regulates translation involve miRNAs, viruses, transposons and plasmid-encoded genes. It is possible that, in the future, other mRNAs that employ a similar mechanism for translational control will be discovered. Alternatively, an interesting gedankenexperiment is that *oskar* might be derived from a transposon itself. It is quite puzzling that *oskar* has no orthologues outside the Holometabola, insects that undergo complete metamorphosis. Also *oskar* mRNA encodes two protein isoforms with differing start codons. Both proteins are translated in a non-stoichiometric ratio with the majority of protein initiated from the second methionine, features that are commonly found in bacterial, but rarely in eukaryotic translation. Additionally, *oskar* mRNA is difficult to express in a cell type other than the nurse cells. Finally, *oskar* is involved in germline formation, a process in which mobile genetic elements are active.

5. Results and Discussion Part II

Characterisation of the oocyte entry signal in the *oskar* 3'UTR RNA.

5.1.Results

5.1.1. A 141bp region is necessary and sufficient for localisation of *oskar* mRNA during early oogenesis.

oskar mRNA is enriched in the posterior half of the oocyte from stages 2 to 7, is transiently detectable at the anterior cortex of that cell stage 8, and is localised to the posterior pole from stage 9 of oogenesis onwards (Ephrussi et al. 1991; Kim-Ha et al. 1991). *Cis*-regulatory sequences involved in posterior localisation were identified previously (Kim-Ha et al. 1993; Hachet and Ephrussi 2004) and recently it was also shown that for early oocyte localisation the *oskar* 3'UTR was sufficient (Jenny, Hachet et al. 2006).

To map the RNA sequences required for this early oocyte enrichment, I expressed *gfp-oskar* 3'UTR reporters in the *D.melanogaster* germ line and detected the RNA by *in situ* hybridisation using a *gfp* antisense probe. The effect of several *oskar* 3'UTR deletions on mRNA localisation was analysed in *oskar* RNA null egg chambers. This genetic background was chosen to prevent any influences the endogenous *oskar* mRNA might have on early localisation of the transgenically expressed RNA. As in *oskar* RNA null flies, oogenesis arrests at stage 7 (Jenny, Hachet et al. 2006), we also expressed the transgenes in wild-type flies to analyse the mRNA localisation within the oocytes of during later stages. Stage 6 oocyte accumulation of transgenic RNA was indistinguishable in *oskar* RNA null and wild-type egg chambers.

Compared to endogenous *oskar* mRNA, the *gfp-oskar* 3'UTR reporter retains full localisation activity (previously described in section 3.2.2). Although the expression levels of the analysed *oskar* 3'UTR reporters was quite similar, we found striking differences in their oocyte accumulation pattern: deletions removing either 5' or the 3' end of the 3'UTR did not affect the stage 6 localisation pattern (constructs 1+2, 2+3), but neither the 5' or the 3' end alone could enrich in the oocyte at this stage (constructs 1, 3). In contrast, the central part of the *oskar* 3'UTR (construct 2) could enrich on its own in the oocyte (Figure 29A). In stage 8 egg-chambers from wild-type flies, the *region2* RNA could further accumulate at the anterior pole at stage 8**,** where it remained detectable until stage 10B (Figure 16**,** section 3.2.6). As all *in situ* hybridisation experiments involved the same *gfp* antisense probe, the localisation differences cannot be due to technical difficulties in detecting the RNA. I therefore conclude that, for early oocyte enrichment, region 2 of the *oskar* 3'UTR is both necessary and sufficient.

This region 2 corresponds to the part of the *oskar* 3'UTR RNA shown to fold into two stem-loops (Christine Brunel, unpublished; this study, section 1.3.3). The second stem is particularly well conserved among *Drosophila* species (see section 3.1). I therefore analysed the oocyte accumulation of the second stem, both in the context of its downstream sequences (region 2b+3), or alone (region 2b). I found that region 2b+3 localises to the oocyte just as the complete *oskar* 3'UTR, when expressed either in wild-type or *oskar* RNA null oocytes. Region 2b so far was not detectable in *oskar* RNA null oocytes, but was enriched in 40% of wild-type egg chambers (Figure 29B). Thus, the 141 nucleotide long central part (region 2b) of the *oskar* 3'UTR seems to contain all sequences necessary for early localisation of *oskar* mRNA.

5.1.2. An AU-rich stem promotes the oocyte localisation of *oskar* 3'UTR.

oskar and *fs(K10)* mRNAs at stage 2-7 are both enriched in the oocyte and at stage 8 they localise to the anterior cortex. I therefore asked whether this similar mRNA localisation pattern is controlled by a similar signal within the RNA sequence. To test this, I fused the non-localising *region3* of the *oskar* 3'UTR to the 44-nucleotide transport/localisation signal (TLS) of *fs(K10)* mRNA (Serano and Cohen 1995). This chimeric RNA reporter could completely recapitulate the early localisation of *oskar* 3'UTR in the oocyte of *oskar* RNA null flies (data not shown). This data indicate that oocyte localisation signals of *oskar* mRNA and *fs(K10)* are interchangeable, as was proposed previously (Serano and Cohen 1995). Furthermore both oocyte localisation regions fold into an extended stem-loop structure. As described already in part I (see section 3.2.5), deletion of the distal part of the second stem-loop (*gfp-oskar* 3'UTR-ΔSLIId) severely reduced oocyte enrichment of the RNA in wild-type egg chambers. This distal region contains the terminal loop involved in direct RNA-RNA interaction and an almost entirely conserved AU-rich stem interrupted by variable bulges (see section 1.3.3.). As a similar AU-rich stem is critical for *fs(K10)* localisation, I therefore next tested what part of this distal stem-loop might serve as the oocyte localisation signal of *oskar* mRNA. To this end, I introduced point mutations in the distal stem, in the context of the SLI-II/region2 and analysed the distribution of the transgenic mRNA, both in egg chambers of *oskar* RNA null and wild-type flies. In each case, stage 6 accumulation of the transgenic RNAs was highly similar in both genetic backgrounds, while, due to the stage 7 oogenesis

arrest, enrichment at stage 8 could only be analysed in wild-type egg chambers. The results are summarised in Table 2.

Deletion of the terminal loop in the context of region 2 did not interrupt *oskar* RNA accumulation at stage 6 or stage 8 (*gfp-oskar* SLI-II-Δloop; Figure 30: A -dloop, B-5'm,3'mand 5'3'm, C-GCstem). This suggests that the palindromic terminal loop that is involved in dimerisation and translation control is dispensable for oocyte entry of *oskar* mRNA.

To determine whether *oskar* mRNA oocyte enrichment depends on the secondary structure of stem-loop II, I introduced point mutations in the most distal six nucleotides designed to disrupt this structure while at the same time maintaining its AU-richness. Both *5'mut* and *3'mut* mRNAs showed a reduced ability to localise in *oskar* RNA null oocytes, *5'mut* mRNA enriching in 24% and *3'mut* in 3% of stage 6 egg chambers (Figure 30B). The 24% enrichment of *5'mut* could be due to a partial formation of a new AU-rich stem predicted by *mfold* for *5'mut* but not for *3'mut* RNAs (data not shown). Restoring the secondary structure by introducing complementary mutations in the distal stem (*5'3'mut*) could partially rescue RNA localisation in stage 6 egg chambers (58%), suggesting that the secondary structure of *oskar* SLII is critical for this RNA localisation (Figure 30B).

I next tested the contribution of AU-richness to localisation, by mutating the most distal six base pairs of the stem into a GC-rich stretch predicted to fold into a stem. Oocyte localisation of this *GCstem* mRNA was reduced (41%), suggesting that both the secondary structure and the AU-richness of the distal stem are critical for oocyte entry of *oskar* mRNA (Figure 30B).

In summary, a 141-nucleotide region of the *oskar* 3'UTR that folds into a stem-loop structure is necessary for oocyte enrichment at stages 2-8. Further, while the terminal loop is dispensable, the AU-richness and, more importantly, the secondary structure of the most distal six base-pairs of the stem are critical for this early localisation of *oskar* mRNA.

Table 2: Summary experiments testing mRNA accumulation in oocytes.

***oskar* mRNA contruct**	**wild-type[1]**		***oskar* RNA null[2]**
	Stage 6	**Stage 8**	
3'UTR-WT	**91**	**97**	**+**
1+2	**+**	**+**	**+**
2+3	**+**	**+**	**+**
1	**-**	**-**	**-**
2	**+**	**+**	**90**
3	**-**	**-**	**-**
2b+3	**+**	**+**	**+**
2b	**+**	**+**	**n.d.**
2-Δloop	**96**	**100**	**89**
2-5'mut	**50**	**14**	**24**
2-3'mut	**44**	**13**	**3**
2-5'3'mut	**72**	**71**	**58**
2-GCstem	**74**	**34**	**41**

+ indicates localisation; - indicates no localisation; Numbers indicate the percentage of oocyte accumulation where scored; n.d., not determined;

[1] ***oskar* reporter mRNA expressed in wild-type egg chambers;**

[2] ***oskar* reporter mRNA expressed in oskar RNA null egg chambers, stage 6 was scored.**

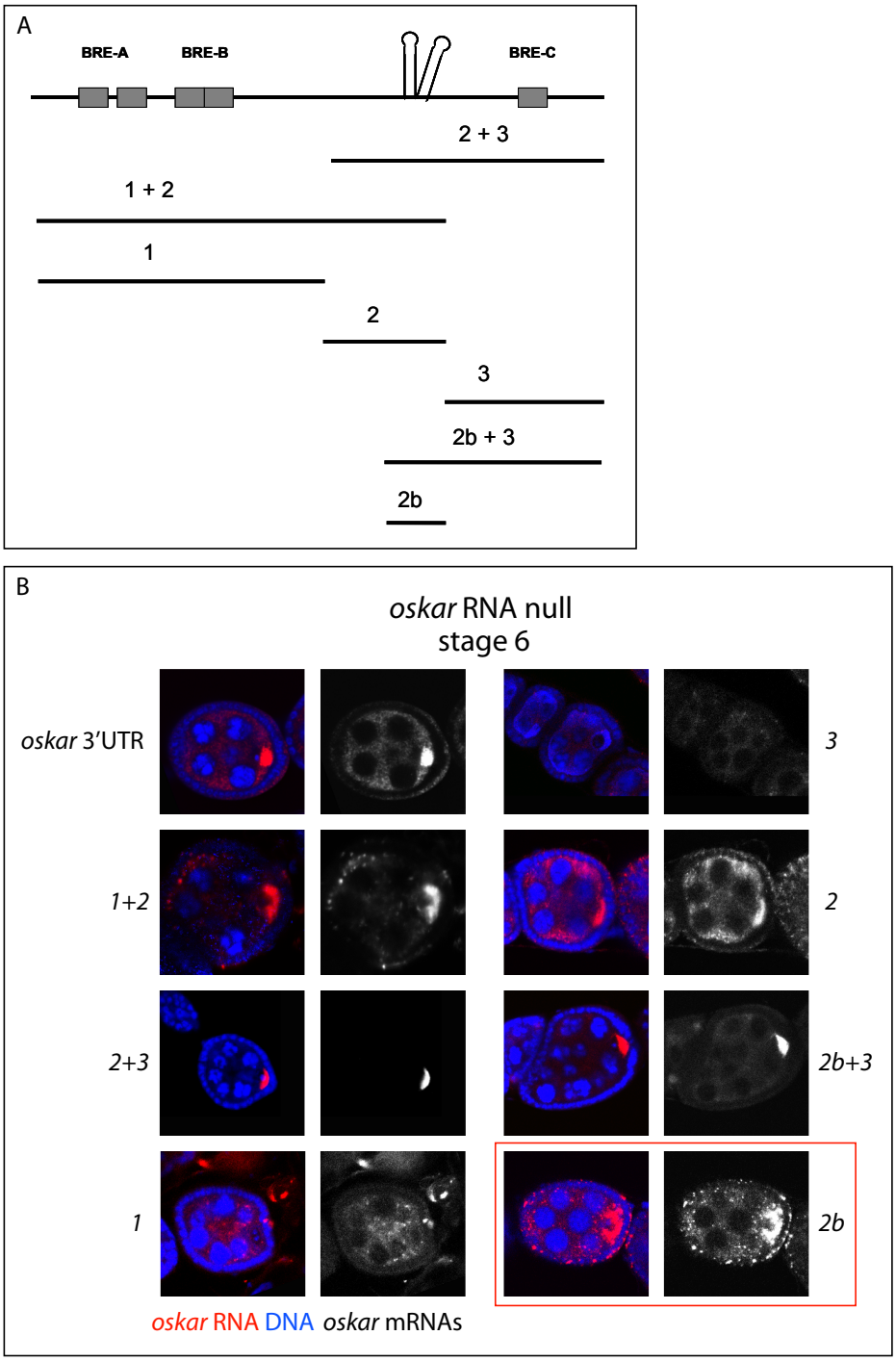

Figure 29: The region 2 of the *oskar* 3'UTR is sufficient for oocyte entry.
A. Schematic of the constructs used to create transgenic flies. A black line represents the 3'UTR regions that were then fused to the GFP open reading frame. For orientation purposes I indicated the location of the Bruno response elements (BREs). Region 2 is identical to the SLI-II fragment that was shown to fold into two stems (C.Brunel). Region 2b is identical to the dimerisation domain characterised in PartI of this work.
B. The accumulation of the *gfp-oskar* 3'UTR RNA fragments was analysed by *in situ* hybridisation using an antisense *gfp* probe. Shown are representative examples of stage 6 egg-chambers from *oskar* RNA null flies. One exception is the analysis of oocyte entry of region 2b that was done in wild-type egg-chambers and is highlighted by a red rectangle.

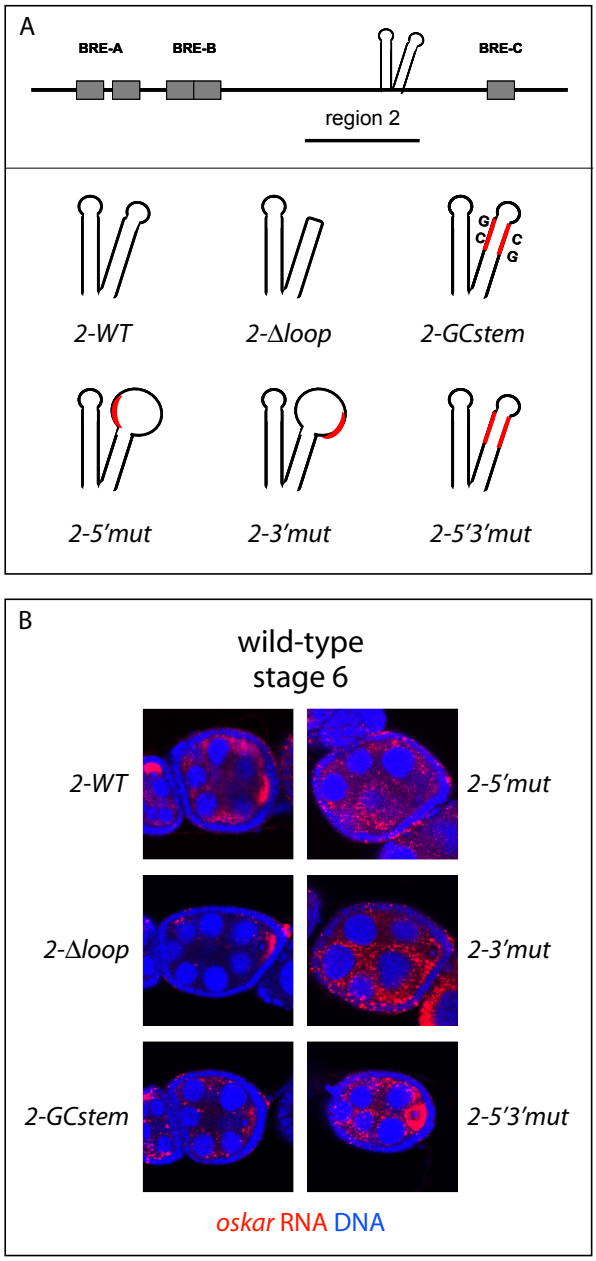

Figure 30: The secondary structure and AU-richness are important for oocyte entry of *oskar* RNA.

A. Schematic of the constructs used to generate transgenic flies. All mutations were analysed in the context of region 2 of the *oskar* 3'UTR and fused to the GFP open reading frame. Parts of the structure shown in black remained wild-type while regions shown in red were mutated.

B. The accumulation of the *gfp-oskar* 3'UTR RNA fragments was analysed by *in situ* hybridisation using an antisense *gfp* probe. Shown are representative examples of wild-type stage 6 egg-chambers.

5.2. Discussion

5.2.1. An oocyte localisation signal for *oskar* mRNA

I have shown that the SLI-II/region2 of the *oskar* 3'UTR is both necessary and sufficient for the early oocyte localisation of *oskar* mRNA at stages 2 to 7. Kim-Ha et al had previously analysed localisation of *oskar* 3'UTR RNAs in egg chambers from wild-type flies and found that deletions in the central part of the *oskar* 3'UTR interfered with anterior enrichment at stage 8 (Kim-Ha et al. 1993). Thus, our analysis of RNA accumulation in *oskar* RNA null flies is consistent with previous observations made in the presence of endogenous oskar mRNA.

Furthermore, I have shown the importance of the secondary structure and AU-richness of SLII for this localisation. However, several aspects of this localisation need to be further investigated. Residual localisation activity remains when the distal part of SLII is deleted. It is currently unclear if the proximal part of the stem contains some capacity to localise to the oocyte, and it is also possible that the *oskar* mRNA oocyte localisation signal is bi-partite. It is also unclear how important AU-richness is, as the GC-rich stem still maintains some localisation activity. This could be due to the one remaining AU-base pair in the GC-rich stem construct, and I am currently testing the effect of a fully GC-rich stem on localisation.

5.2.2. Oocyte localisation: a common signal?

We identified a small part of the *oskar* 3'UTR that contains a signal both functionally and structurally similar to the transport/localisation signal (TLS), of *fs(K10)* mRNA (Serano and Cohen 1995; Serano et al. 1995). These data for the first time show that mRNAs that at stage 9 are found at strikingly different locations in the cell, contain similar localisation signals that direct their early oocyte enrichment.

Several other mRNAs were shown to display similar localisation to the oocyte at stages 2-7, among them *staufen*, *BicD*, *BicC*, *tudor*, *otu*, *cylinB*, and *dacapo* (Suter et al. 1989; Golumbeski et al. 1991; St Johnston et al. 1991; Dalby and Glover 1992; Mahone et al. 1995; Tirronen et al. 1995; de Nooij et al. 2000). Also *orb*, *hts*, *bicoid* and *gurken* mRNAs are similarly enriched, and for these mRNAs the regions directing this localisation were identified. For example a 307 nucleotide long region of the *orb* 3'UTR has full localisation capacity in early oocytes and is predicted to contain a stem-loop similar to the *fs(K10)* TLS

(Lantz and Schedl 1994; Serano, 1995). The *bicoid* 3'UTR contains a 53-nucleotide BLE1 signal, that, when duplicated directs oocyte localisation at stages 2-8 (Macdonald and Struhl 1988; Macdonald et al. 1993). In contrast, *gurken* mRNA contains the signal directing early localisation in its open reading frame; however this element shares some resemblances to the *fs(K10)* TLS (Van De Bor et al. 2005). It will be interesting to see whether early oocyte enrichment of the so far uncharacterised mRNAs also depends on similar signals and, if so, whether these signals share similarities to *oskar* SLII, *fs(K10)* TLS and the *gurken* localisation region.

5.2.3. Current research and Open Questions

As mentioned above, I am further investigating several aspects of the early oocyte enrichment of *oskar* mRNA. Do other, more proximal regions of the stem also contribute to correct localisation? How critical is the AU-richness of the distal stem?

In addition to the similarities between the oocyte localisation signals of *oskar, fs(K10)* and *gurken* described so far, it was also striking that these signals are similar to the apical localisation signals of mRNAs expressed in the *D.melanogaster* embryo, for example *wg, hairy,* and *ftz* (Bullock et al. 2003; dos Santos et al. 2008). Both the oocyte and apical localisation processes have also been shown to require an intact microtubule network (Pokrywka and Stephenson 1995; Lall et al. 1999; Wilkie and Davis 2001; Clark et al. 2007). Furthermore, maternal transcripts could localise apically in embryos when injected as fluorescent RNAs (Bullock and Ish-Horowicz 2001). These parallels between apical transport in embryos and oocyte enrichment in young egg-chambers has been noted previously (Bullock and Ish-Horowicz 2001).

I am therefore currently testing the ability of the SLI-II to enrich apically in the embryo by both *in situ* hybridisation and by injecting fluorescently labelled RNA into embryos. The latter is being done in collaboration with Dr. Simon Bullock (LMB, Cambridge) who initially developed this assay. Dr. Bullock had previously injected *oskar* mRNA transcribed from a cDNA and shown that it could not accumulate apically in embryos (Bullock and Ish-Horowicz 2001). However, it is possible that the much shorter SLI-II RNA (272 nt) will prove to be more appropriate than full-length *oskar* mRNA (2870 nt) for addressing apical enrichment in embryos.

Recent studies in embryos have revealed that ~71% of analysed transcripts are localised in early *D.melanogaster* embryos, and a tempting speculation is that more mRNAs might be enriched in the early oocyte as well (Lecuyer et al. 2007). Together with Evangelia Petsalaki

(group of Rob Russel, EMBL Heidelberg) I am developing an algorithm that could identify such oocyte accumulation/apical localisation signals in all *D.melanogaster* transcripts. In brief, we are searching for nucleotide sequences that are predicted to form an AU-rich stem, interrupted by up to 10 non-complementary nucleotides that would form a loop. We are testing several bioinformatics filters, for example, demanding that the predicted signal be conserved among *Drosophila* species and that it not be located directly at the end of a transcript. Once these are identified, I will test the predictions for a subset by *in situ* hybridisation on oocytes, using the corresponding RNAs as probes. This will allow me to validate the hypothesis, and if successful, may allow a more refined definition of what consistitutes an oocyte localization signal.

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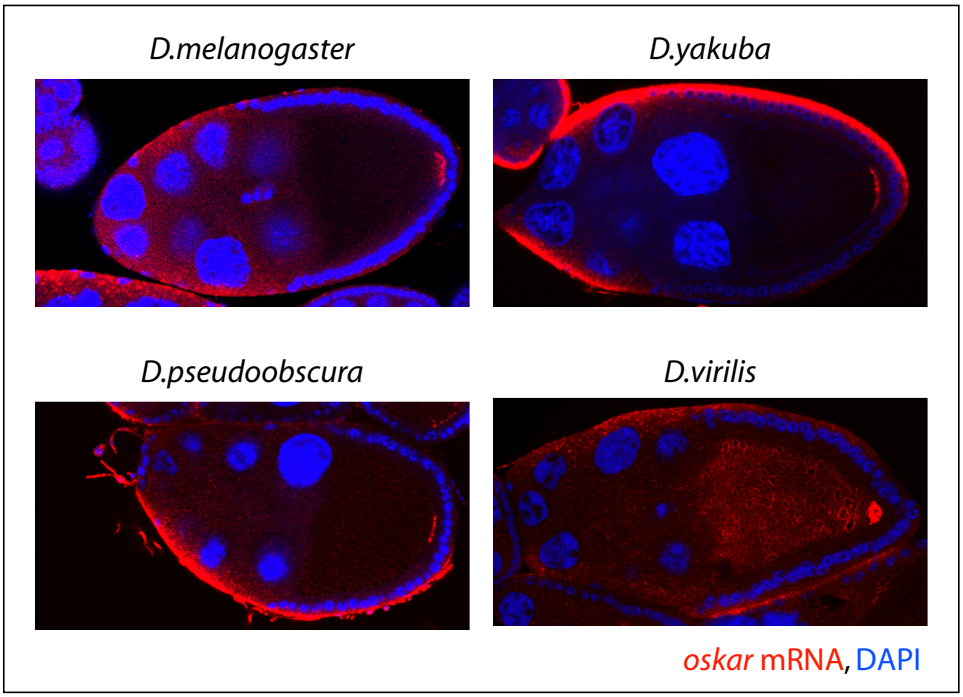
Abbreviations

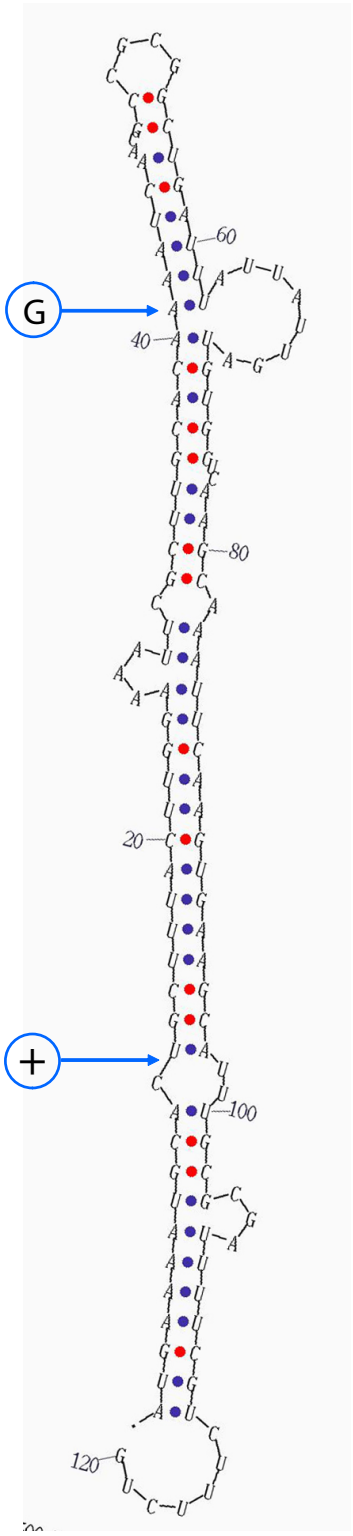
Df	Deficiency
DNA	Desoxyribonucleic Acid
EJC	Exon junction complex
GFP	Green fluorescent protein
Khc	Kinesin heavy chain
mRNA	messenger-RNA
MT	Microtubule
PCR	Polymerase chain reaction
RNA	Ribonucleic Acid
RNase	Ribonuclease
RNP	Ribonucleoprotein
tRNA	transfer-RNA
UAS	Upstream activating sequence
UTR	Untranslated region

Appendices



Appendix I: *oskar* **mRNA posterior pole localisation is conserved between species.** Orthologous *oskar* mRNA (in red) is detected at the posterior pole of stage 9 oocytes from *D.melanogaster, D.yakuba, D.pseudoobscura* and *D.virilis* egg-chambers. *oskar* was detected with antisense probe specific for the each orthologous 3'UTR RNA; DNA was stained with DAPI (blue).

A



Appendix II: The oskar dimerisation domain shows little sequence variation between species (Compare to Figure 9): The secondary structure of the D.melanogaster dimerisation domain is shown. Marked are nucleotides that differ in D.yakuba (A) and D.immigrans (B).

B